Development of an orally relevant biofilm disinfection model

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Abstract

Development of an orally relevant biofilm disinfection model.

This thesis describes the development and use of a novel microtitre plate biofilm system for testing the antimicrobial activity of test materials. The developed model is capable of high-throughput screening and furthermore the system has been shown to be stable and reproducible. The search for new antimicrobial agents for improved plaque control requires appropriate screening models. Key criteria for these models include; predictive of clinical data, orally relevant organisms (mixed species, bacteria present in biofilms), short contact time, rapid, reproducible and high throughput. The most widely used biofilm system for evaluating oral antimicrobials are the Constant Depth Film Fermentor (CDFF) and the Minimum Biofilm Eradication Concentration (MBEC) model systems. Each system has advantages for specific investingations; however neither and no other single system fulfils all of the criteria listed above. The CDFF is an orally relevant model that mimics biofilm development under constant salivary flow; typically the inoculum is derived from human dental plaque. Microbial analyses of *in vitro* growth show populations that are representative of *in vivo* plaque. However, the system is prone to contamination, is labour intensive and has limited capacity for testing multiple agents. The MBEC model investigates the antibacterial susceptibility of attached bacteria to the 96-pegs on the lid of a microtitre plate based system. Unfortunately, this model was not originally designed for use with oral bacteria; therefore, concerns exist for the use of the

MBEC with oral bacteria including the development of oral biofilms on non-orally relevant surfaces such as polystyrene, as found in the MBEC pegs. The aim of this project was to develop a microtitre plate based biofilm assay that could assess the effects of antimicrobials against orally relevant biofilms grown on a relevant surface and compare it to recognised standard model systems. Biofilms derived from a defined inoculum were grown on hydroxyapatite-coated microtitre plate wells. Biofilm characteristics were assessed and were shown to be reproducible and allow for high-throughput screening. Antimicrobial testing showed a dose response and known actives were able to be 'ranked' in the same order as seen in clinical trials. This research has culminated in the development of a simple, high-throughput, reproducible 'off-the-shelf' method for the rapid screening of antimicrobial compounds against an orally relevant biofilm.

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Declaration

I hereby certify that the findings in this thesis result entirely from my own work. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. The basic molecular analysis was carried out in part by Ms. A. Tymon (Eastman Dental Institute, UCL). Colleagues who helped in various aspects of the work are listed in the acknowledgments. This work has not been previously submitted, in part or in full, for a degree or diploma for this or any other university or examination board.

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Special thanks to Kevin for his constant encouragement and support and the making of many dinners.

"A wall has always been the best place to publish your work"

Banksy

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Abbreviations

AB	AlamarBlue
AFM	Atomic Force Microscopy
ATCC	American Type Culture Collection
BA	Blood Agar
BHI	Brain Heart Infusion Broth
CBD	Calgary Biofilm Device
CDFF	Constant Depth Film Fermenter
CFAT	Cadmium Fluoride Acriflavin Tellurite agar
cfu	Colony Forming Units
CHX	Chlorhexidine
CLSM	Confocal Laser Scanning Microscopy
CO_2	Carbon Dioxide
dH_2O	Deionised Water
°C	Degrees Celsius
DNA	Deoxyribose Nucleic Acid
DLVO	Derjaguin Landau Verwey Overbeek Theory

EPS	Extracellular Polymeric Substance
FAA	Fastidious Anaerobic Agar
g	Gram
GC	Gas Chromatography
GCF	Gingival Crevicular Fluid
h	Hours
HA	Hydroxyapatite
L	Litre
Log	Logarithmic
M	Molar
MBEC	Minimum Biofilm Eradication Concentration
MFU	Mean Fluorescence Units
MIC	Minimum Inhibitory Concentration
min	Minute
µg	Microgram
µL	Microlitre
mL	Millilitre

mg	Milligram
mm	Millimetres
MSA	Mitis Salivarius Agar
NCTC	National Collection of Type Cultures
nm	Nanometres
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Solution
ppb	Parts per Billion
PTFE	Polytetrafluoroethylene
RA	Rogosa Agar
R_a	Average Surface Roughness
rpm	revolutions per minute
R+P	Ringers and Peptone Solution
SEM	Scanning Electron Microscope
SPM	Scanning Probe Microscope
sp.	Single Species

spp.	Multiple Species
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
UK	United Kingdom
USA	United States of America
UV	Ultra Violet
VA	Veillonella Agar
VSC	Volatile Sulfur Compounds
v/v	Volume to Volume
w/v	Weight to Volume

Publications and Presentations Related to this Thesis

Chapters in Books

Martin G.C., Burnett G., Middleton A.M., Spratt D., Pratten J. (2007). Effect of Surface Properties on Oral Biofilm Development. Biofilms: Coming of Age (Eds Gilbert P., Allison D., Brading M., Pratten J., Spratt D., Upton M.) pp. 183-191. The Biofilm Club, School of Pharmaceutical Sciences, The University of Manchester, Manchester, UK.

Conference Presentations

Badiei N., Wright C.J., Churchley D.R., **Martin G.C.** (2009). The control of microbial adhesion using sodium tripolyphosphate. Annual Meeting of the International Association of Dental Research, Miami.

Wright C.J., Churchley D.R., **Martin G.C.** (2009). Atomic force microscopy study of the control of microbial adhesion. Annual Meeting of the International Association of Dental Research, Miami.

Martin G.C., Burnett G., Middleton A.M., Spratt D., Pratten J. (2008). Development of Screening Model for Novel Anti-Biofilm Therapies. Meeting of the Scottish Microbiology Society, Glasgow.

Martin G.C., Burnett G., Middleton A.M., Spratt D., Pratten J. (2007). Effect of Surface Properties on Oral Biofilm Development. Meeting of The Biofilm Club, Gregynog.

Martin G.C., Newby E.E (2007). Biological Model for the Identification of Malodour Control Agents. Meeting of the International Association of Dental Research, Thessaloniki.

Conference Posters

Martin G.C., Middleton A.M., Spratt D., Pratten J. (2008). Development of a Microtitre Plate Substantivity Screening Method for Oral Biofilm Disinfection. Meeting of the American Society of Microbiology, Boston.

Martin G.C., Newby E.E (2006). Effect of growth conditions VSC production by oral biofilms. Meeting of the Pan-European Federation Dental Research, Dublin.

Martin G.C., Flanagan A.J., Embleton J., McNab R. (2005). A microtitre plate screening method for oral biofilm disinfection. Meeting of the International Association of Dental Research, Baltimore

CHAPTER 1: Introduction

1.1 Introduction

Do Consumer Healthcare companies need another biofilm model?

Current populations globally continue not only to grow but also increase in age. This is a global challenge brought on by better healthcare provision and diet, moreover, the challenge to ensure a lifetime of full dentition is one currently facing all that work in dentistry. Oral healthcare status is inextricably linked to the physical health of the individual. More importantly, though less investigated, is the effect of dentition on the individual's quality of life. Therefore, it is a challenge for the dental industry and consumer healthcare companies not only to understand possible oral healthcare issues faced by an aging population, but how to, reduce or treat conditions, thus giving patients and consumers a better oral health status for greater quality of 'later' life.

Projections for 2025 suggest the global population expanding from the current 6 billion to over 8 billion, with an average increase in life expectancy to 70-75 years for both sexes, in the next 25 years, according to the United Nations Population Division (United Nations Population Division, 2003). Some developed countries are already reporting average life expectancies to 80 years. A more recent study from the UNPD shows an unprecedented level of increase in 'the proportion of older persons (that is, those aged 60 years or older) are accompanied by reductions in the proportions of children (persons under the age of 15). At the world level, the number of older persons is expected to exceed the number of children for the first time in 2045. In more developed regions, where population aging is far advanced, the number of children dropped below that of older persons

in 1998.' (United Nations Population Division, 2009). It is projected in 2050 that the proportion of the world population 60 years or older will be 22%, with nearly a third in that age bracket in developed countries (United Nations Population Division, 2009). Peterson (2003) discusses how this change in population dynamic will need to be addressed to ensure continual improvement in oral health, in particular focussing on three main points

- Oral health is integral and essential to general health
- Oral health is a determinant factor for quality of life
- Oral health and general health are strongly associated.

The perception of quality of life requires understanding, not only of the tangible factors such as sensitivity, pain, ability to eat and talk, but also those factors which although present in everyone, have effects as individual as the individual themselves, such as the emotional and social aspects of oral health, from appearance and confidence, to self-esteem and social interaction. So the question is, what can we do to help?

As described (United Nations Population Division, 2009) the aged population can be split into three broad but distinct groups,

- The independent
- The frail
- The dependent

Since the poorest healthcare status is in the latter 2 groups, key caregivers are instrumental in the overall wellbeing of the patient/consumer rather than the patient themselves. Therefore, it is paramount to support the primary key care

providers with knowledge and materials to help to continue to support improvements in the oral care status of these two populations, building and supporting the materials provided to and by the dental professional. Interestingly, there is a wealth of material provided for mothers of children, to help in the child's oral health provision, however, at the other end of the scale the material for primary care givers of the elderly is much scarcer. The provision of training and education materials is only one side of the equation, there is an obvious opportunity as people are aging to meet their dental needs now that will secure the best possible dental outcome for their future. This is where most of the dental challenges need to be met.

In the latter part of the 20th century, substantial efforts were made to prevent, or reduce dental caries and these efforts resulted in a marked decline in caries incidence in Western Europe and North America, largely attributed to the use of fluoride in various forms and fluoride toothpastes in particular. Wherever fluoride has been widely used, it has reduced caries incidence substantially. It has been estimated that the ~25% reduction in caries effected, in adults, by the daily use of fluoride toothpastes during many caries clinical trials (Marinho *et al*. 2003, Griffin *et al.* 2007) typically of 3 years' duration. However, caries remains ubiquitous in populations globally; indeed; it is the most common chronic disease worldwide, even in developed market economies, where the use of fluoride toothpastes has been the norm for decades. Although the phrase 'caries prevention' is often used, 'caries postponement' might actually be a better description. Thus, it can be deduced that caries will develop, only later in life.

Therefore with fluoride only achieving half of the equation, it is paramount that the bacteria responsible for caries development and progression be addressed also.

The human oral cavity contains a number of different surfaces each providing a unique physiological environment for bacterial colonisation and biofilm (plaque) development. The physical removal of plaque bacteria by brushing is the main contributor to the maintenance of healthy teeth and gums. Increasing the numbers of bacteria removed and/or killed during brushing is an approach favoured by many healthcare companies via the addition of effective antimicrobial agents to oral care products. Therefore, there is an on going need to identify novel antimicrobial agents that can be incorporated into oral healthcare products e.g. dentifrices and mouthrinses.

The search for new antimicrobial agents for improved plaque control requires that appropriate screening models be put in place. Key criteria for these models include;

- Results should be predictive of clinical outcome
- Orally relevant organisms (including mixed species if possible)
- Bacteria should be present in biofilms
- Short, relevant contact time should be used
- Reproducible methodology
- Rapid and high throughput (~800 screenings/year) capacity

This introduction will therefore consider general biofilms, their importance and why we need to be concerned with them. Moreover, it will focus on oral biofilms

specifically, biofilm diseases, properties of biofilm and how we as researchers can visualise, and model oral biofilms. Finally, the chapter will focus on the pros and cons of these models systems and discuss how the model system that has been developed addresses these deficiencies and subsequently discuss the future possibilities for this model system. Below is an overview of biofilms and their importance in bacterial survival.

1.2 General Biofilm Overview

The survival of microorganisms in various environments is dependent on their ability to attach/adhere to surfaces and subsequently their development into a biofilm. This mode of growth provides bacteria many resistance strategies to antimicrobial challenges and fluctuations in their environment. It is therefore, particularly difficult to remove or disrupt biofilms via either chemical or mechanical means.

1.2.1 Definition of a Biofilm

A biofilm consists of a community of microorganisms enclosed in an extracellular polysaccharide (EPS) matrix which helps retain bacteria at the surface. This mode of growth is distinguishable from planktonic organisms by a number of different characteristic features, including: metabolic collaboration between organisms and co-operation to retain homeostasis (Costerton *et al.*, 1995), cell-to-cell signalling (Cooper *et al.*, 1995) and resistance to antimicrobial agents of up to

concentrations 1000 greater than that required to kill planktonic organisms (Anwar *et al.*, 1989; Lewis, 2001).

1.2.2 Development of a Biofilm

Once cells have attached to a substratum, growth and division occurs and the first step of biofilm development occurs, the formation of micro-colonies as in Figure 1.

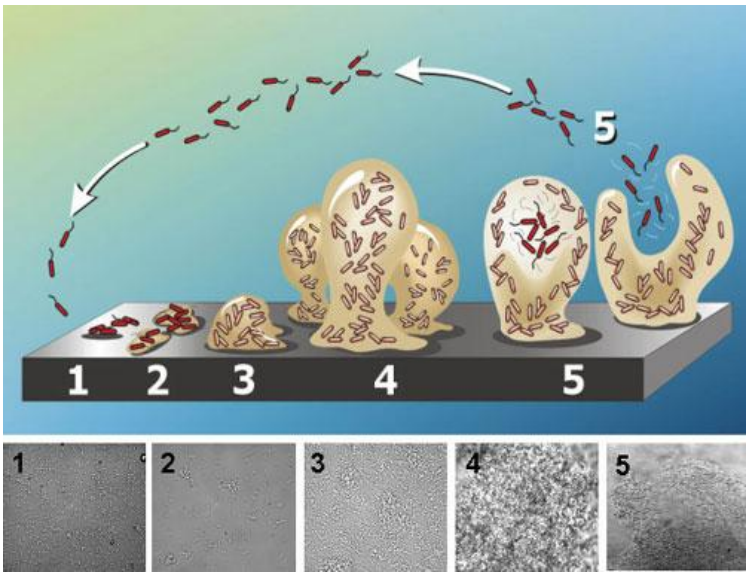

Figure 1. Schematic and micrographs of biofilm development from initial attachment (1), microcolony development (2,3) through to biofilm development (4,5). In this example there is shown release of planktonic cells and re-colonisation (5-1).

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Bacteria will attach to a substratum by either single or multiple processes including ionic interaction, hydrogen bonding (Pimentel and McClellan, 1960) and hydrophobic interactions (Duncan-Hewitt, 1990). These mechanisms are commonly referred to as being non-specific interactions (Busscher and

Weerkamp, 1987). In addition, adhesion to a surface can also be frequently stereospecific, such as the interactions between carbohydrates and lectins (Ofek *et al.*, 1977); these are termed specific interactions. Surface-associated molecules which aid in stereospecific adhesion to a substratum are termed adhesins. Adhesins can be found on a number of bacterial surface associated appendages such as fimbriae, capsules, flagella, outer membranes amongst others (Coutte *et al.*, 2003; Kolenbrander *et al.*, 2006). These adhesins, located at a distance from the main bulk of the cell, enable the bacterium to overcome an energy barrier to adhesion. This barrier was predicted by the DLVO theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). Adhesins of bacteria (may) adhere to receptors on the substratum.

1.2.2.1 Effect of Surface Properties on Biofilm Development

Chemical and physical properties of the underlying substratum can affect the development of an acquired pellicle including its composition, density and configuration. These changes in the physico-chemical properties of the acquired pellicle may influence bacterial attachment and subsequent biofilm development.

1.2.2.1.1 DLVO Theory

The DLVO theory (Derjaguin and Landau 1941; Verwey and Overbeek 1948) approach to bacterial attachment is based upon simple colloidal interactions such as charge and dipole-dipole interactions. Typical force-distance curves derived from DLVO theory, where a positive ΔG represents a repulsive force between the surface and bacterium is shown in Figure 2. These force-distance curves are the

product of two separate competing forces, that of van der Waals attraction and ionic repulsion. Hence, the overall force curve is dependent upon the relative contributions of these separate forces. At large separations between the bacterium and the surface, there is no net attractive or repulsive force acting. However, as the surface and bacterium approach there is a mild attractive force generated from van der Waals forces (dipole-dipole interactions). The strength of this attraction increases in proportion to the reciprocal of the separation distance as the bacterium approaches the surface in accordance with Hamaker theory (1937), leading to a net attractive force at the secondary minimum. While the importance of the secondary minimum to weakly flocculated colloid gels has been well described, its role in bacterial attachment to surfaces is relatively poorly understood (Redman *et al.*, 2004). At smaller separations, the overlap of the diffuse double-layer charges of the surface and the bacterium can have a repulsive effect if (as is often the case) the bacterium and the surface are both similarly negatively charged.

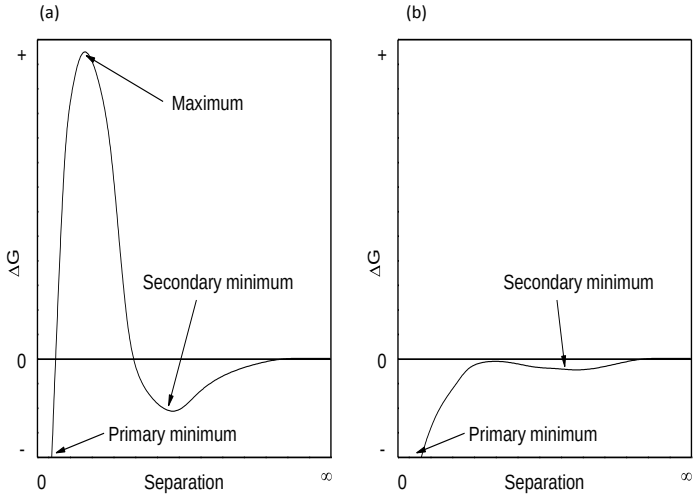


Figure 2. Schematic of DVLO curves for systems with high (a) and low (b) electrostatic repulsion

This repulsion tends to be relatively strong at medium separation compared to the van der Waals attraction and generally leads to a prominent repulsive maximum element in the DLVO curve (fig 2a) restricting the ability of the bacterium to approach the surface. If the bacterium has sufficient energy to overcome this electrostatic repulsion barrier there is, at short separations, a net attraction (the primary minimum) between the bacterium and surface which will lead to contact of the two. It should be noted that this attachment process is driven entirely from an energetics perspective and as such is a reversible process. Further it describes idealised 'simple' cells and surface, and thus doesn't consider adhesion-receptor interactions.

1.2.2.1.2 Surface Properties

Most bacterial cells display a net-negative charge at neutral pH (Jucker *et al.*, 1996), although this charge is usually reduced as the pH is lowered owing to neutralisation of acid groups on the surface of the bacteria (Hayashi *et al.,* 2001). This negative charge will hinder bacterial attachment to negatively charged surfaces due to charge-charge repulsions. Most surfaces are negatively charged in aqueous environments as a consequence of cations being generally more solvated than anions which are more generally associated with the surface (Shaw, 1986). Therefore, in most cases at neutral pH there is a net electrostatic repulsion between the surface and the bacterium leading to DLVO curves similar to fig 2a. However, if the pH is lowered to below the pKa of the bacterium or surface (Horka *et al.,* 2006) the electrostatic repulsion will diminish, leading to curves

more similar to fig 2b, where van der Waals attractions dominate (Abu-Lail and Camesano, 2003). Similarly, the electrostatic repulsion may be strongly reduced by increasing the ionic strength of the solvent. In the case where the charge of the bacterium is positive, strong attraction to negatively charged surfaces has been observed under conditions where negatively charged bacteria were still repelled from the surface (Jucker *et al.*, 1996). Work has also shown bacterial adsorption could be increased on negatively charged polymethyl methacrylate (PMMA) surfaces, by varying the concentration of a positively-charged co-polymer (Kiremitci-Gumusderelioglu and Pesmen, 1996).

Of most interest in the development of model systems is where polyelectrolytes or surfactants (Olsson *et al.*, 1991) are physisorbed to the surface so reversing or reducing the charge of the surface. Of particular concern for oral biofilm models is the role of the salivary pellicle, a proteinacious layer that can be detected within 30s of placing clean enamel in saliva (Vacca Smith and Bowen, 2000). This pellicle effectively reverses the charge of the surface since positively charged proteins provide the initial attachment to the surface. The pellicle can therefore reduce the repulsive aspects of the DLVO force-curve, promoting bacterial adhesion (Shimotoyodome *et al.*, 2007). Hydrophobicity of the surface can also affect bacterial adsorption, either directly or by affecting the adsorption of a proteinaceous pellicle (Sethuraman *et al.*, 2004). Tegoulia (2002) showed that adhesion of *Staphylococcus aureus* to surfaces generally increased as the hydrophobicity of the surface was increased. Triandafillu and co-workers (2003) showed that treatment of a PVC surface with oxygen plasma reduced its

hydrophobicity and subsequently reduced bacterial adhesion. These hydrophobic effects have been rationalised by Rijnaarts *et al.* (1999) as due to reduced reversibility of the bacterial adsorption. Unlike hydrophilic surfaces, once a hydrophobic bacterium adhered to a hydrophobic surface it was not able to detach. Such strong binding of bacteria to hydrophobic surfaces can be explained by a hydrophobic dehydration mechanisms (Norde and Lyklema, 1991).

The physical properties of surfaces have also been investigated to determine the effect of topography on bacterial attachment (Quirynen *et al.,* 1990; Taylor *et al.*, 1998), retention (Nyvad and Fejerskov, 1987), subsequent biofilm development (Pratten *et al.,* 2003) and susceptibility to test agents (Korber *et al.,* 1997). The effect of surface roughness has been evaluated for its effect on food processing (Korber *et al.,* 1997), denture and implant manufacture (Taylor *et al.,* 1998) and dental plaque development (Bollen *et al.*, 1997; Quirynen *et al.*, 1990; Charman *et al.,* 2009). Figure 3 shows an increase in *Streptococcus oralis* attachment to acrylic with increase in surface roughness.

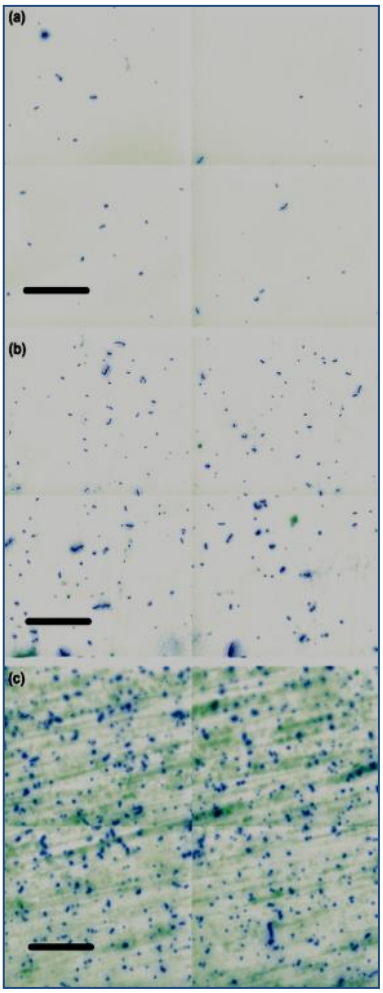


Figure 3 Photomicrographs showing bacterial attachment on acrylic samples with differing R_a values, after 60 min incubation. (a) R_a 0·07 µm; (b) R_a 0·38 µm; (c) R_a 1·14 µm. Scale bar = 50 µm (Charman *et al.*, 2009)

Surface roughness (R_a) is usually characterised as the sum of departure of the mean of the profile from the mean line (see Figure 4).

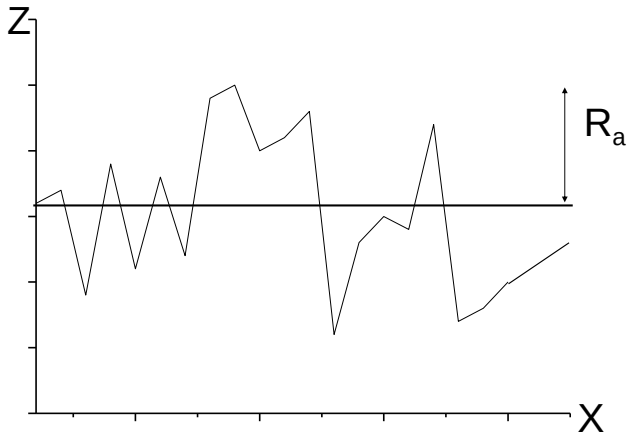


Figure 4. Schematic of surface topography where R_a = arithmetic average roughness.

To fully understand the implications of R_a on bacterial attachment, the surface area should also be taken into consideration. Figure 5 shows two graphs which have the same R_a, however, the surface areas are very different. Increased surface area may assist the retention of organisms on these surfaces due to the increase in contact points (Whitehead *et al.*, 2006).

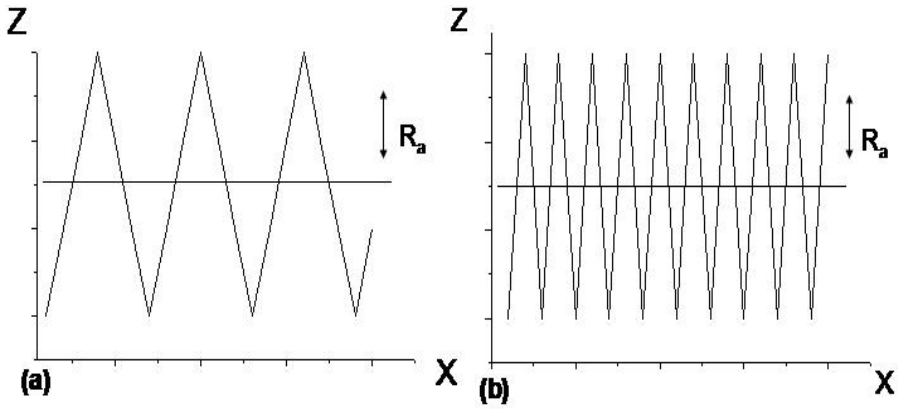


Figure 5 Different surface areas may produce the same R_a. In this example image (a) has the smaller surface area than (b), but both still have the same R_a value.

There are a number of key parameters that will determine biofilm attachment, retention, subsequent growth and potential effects. Below is a summary of the effects of biofilms in medicine. To understand the biofilm structure is a potential route to understanding a treatment against it.

1.2.2.2 Biofilm Structure and Form

In vitro biofilm structure is determined by available nutrients, substratum, organisms present and incubation conditions. Changes in any one of these parameters will affect bacterial growth and biofilm development, thus affecting biofilm structure. Co-aggregation is a specific bacteria-to-bacteria adhesion which plays a critical role in the development of a multispecies biofilm. Coaggregation is controlled through specific adhesion interactions (Rickard *et al*. 2000; Rickard *et al.* 2004) with planktonic cells showing a decrease in coaggregation compared with their biofilm counterparts (Rickard *et al.* 2003). This offers an advantage metabolically, as Bradshaw *et al.* (1998) discovered to obligate anaerobes living in an oxygen containing environment. Using fluorescent *in situ* hybridisation (FISH) to determine the location of bacteria held within the EPS matrix (Costerton *et al.*, 1995) in conjunction with confocal scanning laser microscopy to reveal the overall structure (Auschill *et al.*, 2001; Wood *et al.*, 2000), it has been shown that biofilms are highly structured and contain water channels, which have been proposed as a simple circulatory system (Costerton *et al.*, 1995). This open system allows transport of nutrients and solutes to the bacteria and can excrete molecules away from the cell. The most viable cells are found to be lining these voids and channels (Auschill *et al.*, 2001).

It is reported that EPS matrix forms 75-80% volume of the biofilm (Socransky and Haffajee, 2002). EPS plays a prominent role in bacterial retention at a surface. Not only does the EPS form the sticky matrix that can ultimately regulate the structural component of the biofilm, but it may also affect initial attachment (see Figure 6). Tsuneda (2003) compared attachment of 27 bacterial strains correlating those expressing high EPS with greater adhesion onto glass beads. The authors ascribe this effect as being due to the polymer interacting between the surface and the bacterium like a tether. Such polymeric interactions, often referred to as steric interactions, can cause strong attractive bridging when the polymer has a high affinity to the surface. It is estimated that of the total organic carbon content of biofilms, 50-90% comes from the EPS (Flemming *et al.*, 2000) with 40% of the dry weight of dental biofilms being polysaccharide in nature (Paes Leme *et al.*, 2006).

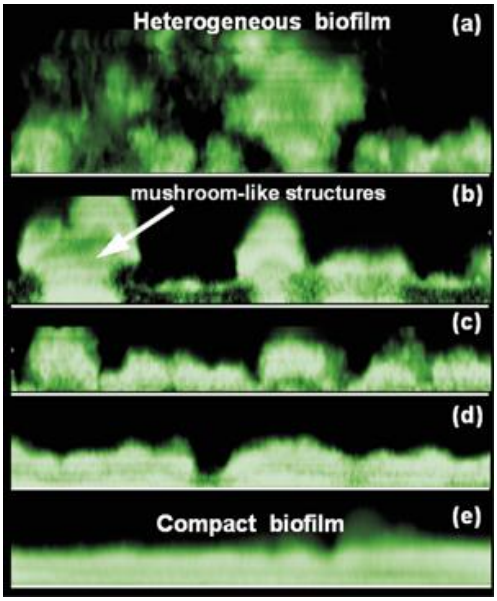


Figure 6. The morphology of a biofilm can vary from a compact and smooth layer to a very diffuse layer, depending on a number of factors such as shear, nutrient availability (image taken from http://www.delftoutlook.tudelft.nl/info/indexe0ee.html)

Hence to evaluate the efficacy of novel test agents, relevant biofilms should be employed to determine the active concentrations which may be required to eliminate the organisms growing within these structures, as it is well reported that the formation of biofilms by the organisms confers increased antibacterial resistance.

1.2.3 Antimicrobial Resistance of Biofilms

Biofilms have been shown to be more resistant when under an antimicrobial challenge compared with planktonic cultures of the same organism (Costerton *et al.*, 1995; Larsen and Fiehn, 1996; Wilson, 1996). This has been found to be as much as 1000-fold increase in resistance (Mah and O'Toole, 2001). Biofilms have shown responses including incomplete penetration of antimicrobial agents through the biofilm, which prevents the antimicrobial from reaching its target, reduced metabolism of bacterial cells due to changes in microenvironments, slower growth rate and expression of novel phenotypes at the surface (Mah and O'Toole, 2001; Stewart and Costerton, 2001; Szomolay *et al.*, 2005).

Current reasoning behind why biofilms are more resistant to antimicrobials than their planktonic counterparts focuses on two mechanisms, either innate or adaptive (Anderson and O'Toole, 2008). It is postulated by Anderson and O'Toole that induced resistance has been shown to exist within biofilms, however, the main protective mechanisms seem to be innate defences. Interestingly, it is known that bacteria which are found in a biofilm can exhibit different phenotypes. As stated by Roberts and Mullany (2010), "Most pathogens

do not intrinsically contain resistance genes, as has been demonstrated by the numerous completed genome sequences of pathogenic bacteria, and it is only the acquisition of such genes that leads then to become the multiple antibiotic-resistant bacteria that are the scourge of modern healthcare." Therefore, understanding the transfer of these resistance genes within biofilms is of great interest to help reduce, and prevent biofilm re-growth.

1.2.3.1 Innate Response - Incomplete Penetration

Penetration of antimicrobials through the whole biofilm structure is paramount for the destruction of all viable bacterial cells and preventing biofilm regrowth. As discussed previously, biofilms are covered in an extracellular polymeric substance (EPS). This material, although containing a number of different materials, such as DNA and protein (Donlan and Costerton, 2002), is largely negatively charged and very effective at reducing the penetration of positively charged antimicrobials, potentially via electrostatic binding (Ishida *et al.*, 1998; Shigeta *et al.*, 1997). Retardation of the antimicrobial within the matrix might develop concentration gradients across the biofilm. Thus, there is the potential to retain the antimicrobial/antibiotic at sub-MIC levels towards the 'core' of the biofilm which has been studied widely (Suci, 1994; Donlan and Costerton, 2002; Walters *et al.*, 2003). Interestingly, in a number of studies, it was observed that the leading biofilm edge showed reduced viable bacterial numbers. However, even with the penetration of the test agent throughout the biofilm, bacterial viability was not drastically impacted (Walters *et al.*, 2003; Zheng and Stewart, 2002).

Furthermore, it should be noted that some researchers have identified a number of antibiotics which can increase EPS formation. Rachid *et al*. (2000) showed that sub-inhibitory concentrations of erythromycin, tetracycline and quinupristin-dalfopristin caused *Staphylococcus epidermidis* gene expression of the polysaccharide intracellular adhesin to be activated; the effect of this was seen in microtitre plates which revealed an increase in biofilm formation. This mechanism seems to differ, dependent on the organism under investigation. Up-regulation of alginate expression was seen when *Pseudomonas aeruginosa* was exposed to sub-inhibitory concentrations of imipenem (an intravenous β-lactam antibiotic) (Bagge *et al.*, 2004b). This raises an interesting predicament as discussed by Anderson and O'Toole (2008); "It is intriguing to speculate that limited antibiotic diffusion through the biofilm matrix, coupled with a corresponding decrease in antimicrobial concentration might actually stimulate the biofilm formation in some instances by creating a positive feedback loop."

1.2.3.2 Innate Response - Persister Cells

Persister cells were first described in 1944 (Bigger, 1944) as a mechanism for survival, however, the mechanism itself is still not fully understood. Bigger found that when treated with lethal concentrations of penicillin, a small fraction, found to be 1 in 10 of the colony forming units of the original concentration or less, of *Staphylococcus aureus* survived. Surprisingly, if these organisms were cultured and exposed to the penicillin again, they had reverted to being sensitive to the antibiotic. Little research was carried out in this area until recently, when a relationship between the growth rate of the organisms and antibiotic sensitivity

was found (Trumanen *et al.*, 1986; Aridesi *et al.*, 2003). Interestingly a mathematical model was employed by Roberts and Stewart (2004) to investigate the effect of antibiotic challenge on biofilms with respect to changing localised nutrients and growth rates. This supported and confirmed the findings that nutrient availability and growth rates may be critical in the resistance to antibiotic challenges. However, evidence does exist to show that persister cells can survive even after exposure to ofloxacin, which has been shown to kill non-growing organisms (Kaldalu *et al.*, 2004), therefore, concluding that slow growth rates cannot be the only survival mechanism at work for persister cells.

1.2.3.3 Adaptive Responses

Environmental stresses have been investigated for their effect on biofilms and planktonic cultures with respect to stress-response genes. This work has shown that the organisms can revert to more tolerant phenotypes under stress such as low nutrients, cell density, pH, temperature and osmolarity (Novick, 2003). There is mounting evidence that biofilms can demonstrate different adaptive responses to antimicrobial stress (Szomolay *et al.*, 2005). These adaptive responses include an increase in EPS synthesis in response to antimicrobial challenge (Sailer *et al.*, 2003), as previously discussed. An example of a specific response is an increase in catalase production by *P. aeruginosa* during exposure to 50mM hydrogen peroxide (Elkins *et al.*, 1999; Sailer *et al.*, 2003). Similarly, β-Lactamase production and potentially efflux pumps may help biofilms during an antimicrobial challenge (Anderl *et al.*, 2000; Bagge *et al.*, 2004a, 2004b; Denkard and Ausubel, 2002). However, these systems have been disregarded as important

in biofilm resistance but do play an active role in planktonic cell antibiotic resistance (Patel, 2005; Stewart and Costerton, 2001).

1.2.3.3.1 *Genetic Variability*

Roberts and Mullany, (2010), present a clear and concise overview of the mechanisms of gene transfer, this can be found in Table 1.

Table 1. Mechanisms of gene transfer among bacteria (modified from Roberts and Mullany, 2010)

Mechanisms used in DNA transfer between cells - Transformation - Conjugation - Transduction
Transformation: Uptake of available DNA from the surrounding environment. **Conjugation:** Sharing of DNA between viable cells **Transduction:** DNA taken up by a bacteriophage subsequently deposited in a secondary cell.

A second key consideration which aids gene transfer is the close proximity of mixed species in a biofilm (Roberts and Mullany, 2010). The extent of this exchange of genetic information was discovered in a study carried out by Lancaster *et al.* (2003) and Ready *et al.* (2003) on children's oral microbiota where antibiotic resistance genes could be identified. Lancaster found that 98% of children in their study carried tetracycline-resistant bacteria.

When grown in a biofilm, Yamanake *et al.* (2009) showed that *Prevotella intermedia* strains showed different genes to be either positively or negatively regulated in comparison to when grown planktonically. Even within a biofilm, bacteria can express the same genes at different levels (van der Woude, 2005). Such switching offers a route to evade, in this instance, the immune system (Deitsch et al. 1997). Phase variation, as this is termed, can not only be used by organisms to evade the immune response. *Escherichia coli* outer membrane protein, Ag43, aids in biofilm formation (Pratt and Kolter, 1998; Danese *et al.*, 2000; van der Woude and Baumter, 2004). Subsequent studies show that if Ag43 was affected by phase variation, biofilm formation was impacted (Schembri and Klemm, 2001; Wallecha et al. 2003; Holden and Gally, 2004). Therefore, this raises the question, if indeed biofilms offers better protection against external challenges, what is the benefit of phase variation of Ag43 if it places the cell, potentially in the path of the immune system.

As discussed above, all these points above require consideration when trying to address biofilms, in particular those associated with disease. Bacteria have developed a number of routes to evade either the immune system or chemical and physical removal. This is of course a concern as the impact of biofilms economically, physically, and less studied, mentally is immense.

1.2.4 Importance of Biofilms

Medical biofilms can increase the morbidity and mortality of affected individuals and are usually found either associated with medical implants (Donlan and

Costerton, 2002) or disease states such as open wounds, cystic fibrosis, colitis, vaginitis, urethritis, conjunctivitis and dental plaque to name a few (Chernish and Aaron, 2003; Holby *et al.*, 2005). In 2007, The Centers for Disease Control and Prevention Report stated "In American hospitals alone, healthcare-associated infections (i.e. nosocomal infections) account for an estimated 1.7 million infections and 99,000 associated deaths each year. Of these infections: 32% of all healthcare-associated infections are urinary tract infections; 22 per cent are surgical site infections; 15 per cent are pneumonia (lung infections); and 14 per cent are bloodstream infections". Persistence of biofilms, as discussed previously has serious implications for the patient due to the decrease in antibiotic susceptibility. Interestingly, some of the topics discussed previously, where the biofilm may, as a consequence of exposure to an antibiotic, increase biofilm volume (Bagge *et al.*, 2004b). This would be detrimental to, for example, a patient suffering cystic fibrosis.

In this next section we will briefly cover the effect of oral biofilm, where the economic, social and personal burden can be challenging. It is reported by the World Health Organisation, that oral disease is the 4th most expensive disease to treat in industrialised countries, with 60-90% of school children globally suffering dental caries (WHO Factsheet 318, 2007). In England alone, in 2006 the National Health Service spent £1977M in dentistry for dental treatments and disease prevention, this equates to £39 per head of population (NHS Report, 2008).

Therefore, having the ability to efficiently and effectively screen actives against orally relevant biofilms is a key challenge for industry to help address patient's and consumer's oral care needs, before treatment is sought.

1.3 Oral Biofilms Overview

The human oral cavity contains a number of different surfaces each providing a unique environment for bacterial colonisation and biofilm development. Molecular techniques have now identified over 630 different bacterial species in the oral cavity (Kazor *et al.*, 2003), and more recently using 454 pyrosequencing Zaura *et al.* (2009) identified over 3600 unique sequences within an individual oral cavity.

The main surface that harbours the bulk of the oral bacterial numbers is the tongue, with its unique papillary structure and large surface area (Jacobson *et al.*, 1973), estimated at approximately 107.5cm^2 (Collins and Dawes, 1987) or 50% of the total oral cavity surface area. In contrast, it is estimated that the teeth comprise 20% of the oral cavity surface area (Collins and Dawes, 1987). The tooth/gum structure also allows the development of two further distinct environments, supragingival (above the gingival margin and interdental) and subgingival (below the gingival margin in the gingival pocket).

Bacterial colonisation of these environments is selective due to the expression of specific adhesins that are complementary to different receptors found at these sites (Gibbons *et al.*, 1976b; Gibbons, 1989). This specificity leads to the development of specific biofilms associated with each of these environments, which if left untreated, can lead to the development of site specific oral conditions. The oral cavity is inevitably prone to colonisation by microorganisms because of its warm, moist environmental conditions. However, under normal/healthy conditions, no

invasive infections of the tissue occur (Dommisch *et al.*, 2005). This is due, in part to the defence mechanisms of the oral epithelium. The oral epithelium acts as a barrier to infection. It also produces antimicrobial peptides called defensins of which there are two classes, α and β. Defensin production is not exclusive to the oral epithelium; they are produced by epithelial cells of all mucous membranes in the body. These peptides have a high number of basic amino acid residues which give them a positive charge (Dommisch *et al*., 2005). Defensins form aggregates within the membrane of the organism, resulting in formation of pores. This results in osmotic shock and ultimate death of the pathogen, (Dommisch *et al.*, 2005). Three subtypes of human β-defensins, (HBD), have been identified to date, called HBD -1, -2, -3. HBD-1 has antimicrobial activity against gram-negative bacteria, (Goldman *et al*., 1997), HBD-2 has antimicrobial activity against gram-negative bacteria and some fungi (Harder *et al*., 1997), while HBD-3 has antimicrobial activity against both gram-negative and gram-positive bacteria (Harder *et al*., 2001).

1.3.1 Supragingival Plaque

Supragingival plaque is the result of biofilm development above the gingival margin on the tooth surface (this also includes interdental biofilms). As this surface, unlike any other in the body, is non-shedding, therefore, physical and chemical means are required to remove accumulated plaque Table 2 shows the proportions of bacteria found in accumulating supragingival plaque and how the population changes over time.

Table 2 Supragingival plaque – Proportions of bacterial species over time (Marsh and Martin, 1992)

Bacterium	Hours of plaque development		
	2	4	6
S. sanguinis (formerly known as *S. sanguis*)	8	12	29
S. oralis	20	21	12
Mutans streptococci	3	2	4
S. salivarius	<1	<1	<1
Actinomyces viscosus	6	7	5
A. naeslundii	1	1	3
A. odontolyticis	2	3	6
Haemophilus spp.	11	18	21
Capnocytophaga spp.	<1	<1	<1
Fusobacterium spp.	<1	<1	<1
Black-pigmented anaerobes	0	<0.01	<0.01

Within the first 24-hours of colonisation, oral streptococci have been shown to make up 60-90% of supragingival plaque (Mager *et al.* 2003; Nyvad & Kilian, 1987). However, this is contradicted by Li *et al.* (2004), who found that during early biofilm formation *Actinomyces* spp. are predominant. Interestingly, where *Streptococcus mutans* is often discussed as the main aetiological agent associated with the development of caries (Kreth, 2005; Marsh, 1995b), *Streptococcus salivarius* is often associated with building stability in the biofilm matrix via lactose uptake and urease enzymes (Chen *et al.*, 1996; Sissons and Yakub, 2000) and with this stability prevent colonisation by opportunistic pathogens (Tada *et al.*, 2006; Uehara *et al.* 2001).

Initial bacterial colonisers do not adhere to the tooth surface, rather they adhere to the salivary / dietary derived conditioning film (Dawes *et al.*, 1963). In the oral

cavity this conditioning film and the molecules contained within it act as receptors for bacteria to attach (Gibbons *et al.*, 1976a; Gibbons, 1989). This pellicle contains components from the shedding mucosa, saliva and diet and includes lysozyme, glycoproteins, phosphoproteins, albumin, lipids and gingival crevicular fluid (Marsh, 1995a; Teughels *et al.*, 2006). The conditioning film affects the order of microbial colonisation (Marsh, 2004). Figure 7, shows the localisation of bacteria within supragingival plaque, with respect to the tooth surface.

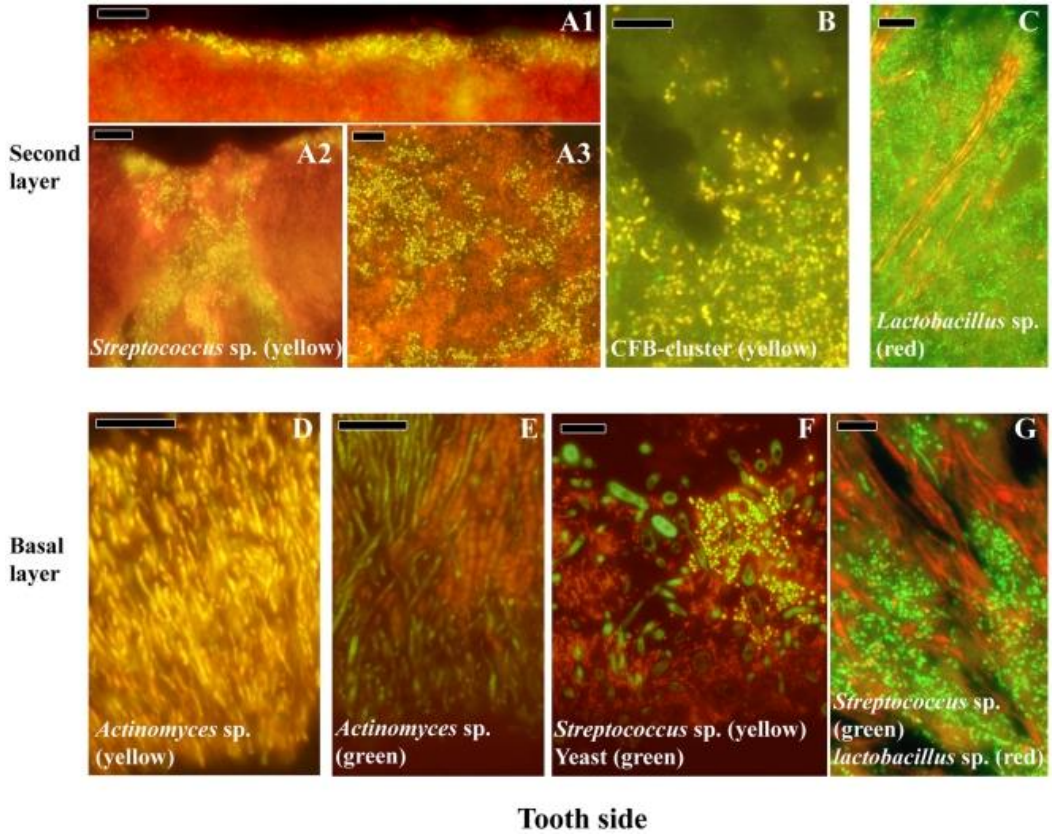


Figure 7 Localisation of the main bacteria found in supragingival biofilms. Bacteria double stained with probe EUB338 labelled with FITC or Cy3. Bars are 10 µm , taken from Zijnge (2010). Image highlights different stratification that occurs within supragingival biofilms from the tooth out towards the oral cavity.

1.3.1.1 Caries

In the latter part of the 20th century, substantial efforts were made to prevent or reduce dental caries. These efforts resulted in a marked decline in caries incidence in Western Europe and North America, largely attributed to the use of fluoride in various forms and fluoride toothpastes in particular.

As discussed previously, Li *et al.*, (2004) found that during early biofilm formation, *Actinomyces* spp. are predominant. However, the microbiota shifts during colonisation and at 24 hours, streptococci make up 60-90% of dental plaque (Marsh and Martin, 1999; Nyrad and Kilian, 1990). It has been shown that *S. mutans* is able to metabolise dietary carbohydrates to organic acids such as pyruvic or lactic acid (Cotter and Hill, 2003), which in turn can lead to the development of carious lesions on enamel surface (Marsh, 1995b). However, *S. mutans* is not the only aetiological agent associated with caries. Various *Lactobacilli* sp. along with *S. sanguinis* some *Actinomyces* sp., and enterococci (Loeche, 1986; Ikeda *et al*., 1973; Nikiforkuk, 1985) have been shown to cause demineralisation of the tooth enamel, the initial stages of caries formation. Caries incidence has also been linked to flow rates, pH, saliva buffering capacity, calcium, available protein and saliva antioxidant levels (Preethi *et al.*, 2010).

As with coronal caries, caries on root surfaces is also ubiquitous where root-surfaces are exposed (Beck, 1993; Nunn, 2000; Stamm *et al.*, 1990, Fejerskov *et al.*, 1993; Fure and Zickert, 1990; Splieth *et al*., 2004; Kularatne and Ekanayake, 2007). It seems likely that individuals who have experienced coronal caries will

go on to develop root surface caries (Beck and Drake, 1997; Burt *et al*., 1986). The findings of a recent US survey suggest that older adults experience high rates of new caries (Griffin *et al*., 2004) and root-caries rates in adults can approach caries rates of children (Beck, 1993). When caries susceptible root and coronal surfaces were compared, the rate of root caries was almost double that of coronal caries (Hand *et al*., 1988). Although often considered a disease of the elderly, relatively high numbers of individuals in the 30-50 age-groups were found to have untreated carious root lesions (Fejerskov *et al*., 1993). Therefore, prevention of caries is a lifetime challenge, especially as people are living longer.

1.3.2 Subgingival Plaque

Subgingival plaque is formed in the protected environment between the gingival margin and the tooth surface. Due to this location, the main nutrition comes from gingival crevicular fluid (GCF), as well as potentially blood. An anaerobic environment can develop and lead to a predominance of asaccharolytic species (Darveau *et al.*, 1997). GCF has been shown to contain a number of antimicrobial components as part of the innate immune response, which include lysozome, complement and vascular permeability enhancers such as bradykinin, thrombin and fibrinogen (Darveau *et al.*, 1997). Figure 8 shows the stratification of the organisms that can occur in subgingival plaque within the periodontal pocket.

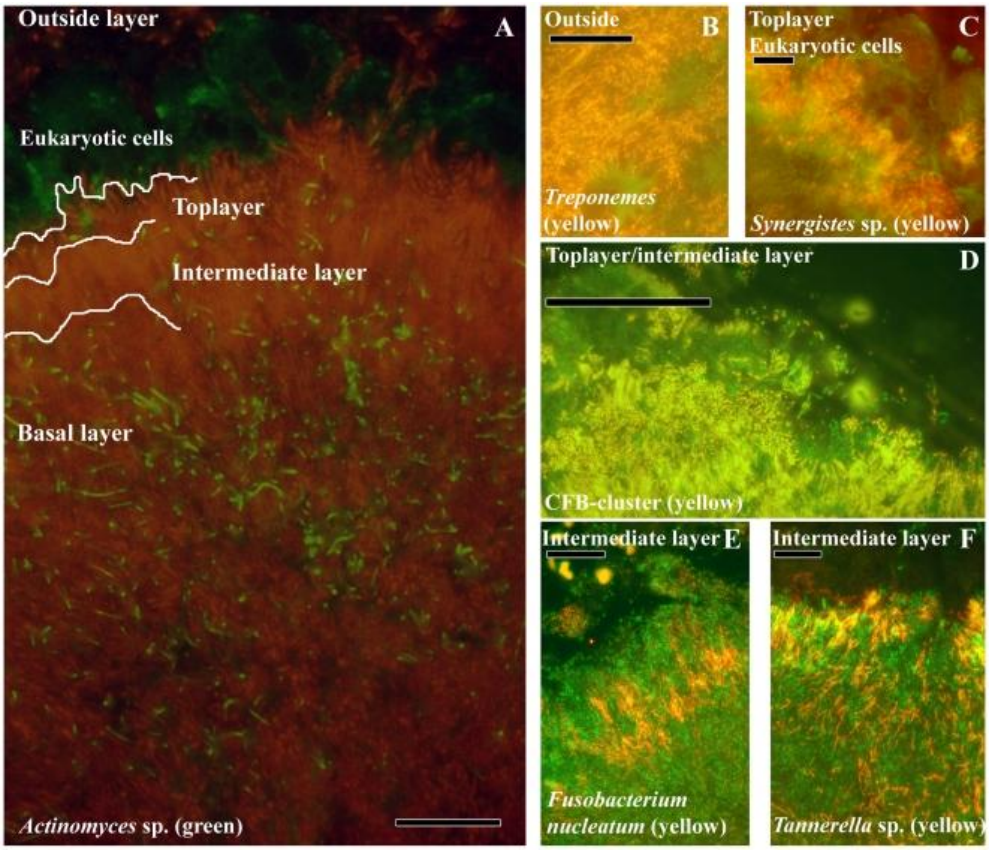


Figure 8 Showing the stratification and location of commonly found organisms in subgingival plaque. Biofilms were double-stained with probe EUB338 labelled with FITC or Cy3. The yellow colour results from the simultaneous staining with FITC and Cy3 labelled probes. Bars are 10 µm, taken from Zijnge (2010). Showing stratification that occurs from the base of the sulcus towards the gingival margin.

According to Socransky *et al*., (1998), this complex microbiota can be further defined into clusters of organisms; a diagrammatic representation can be found in Figure 9. The authors (Socransky *et al.,* 1998) discuss that their term 'red complex' microbiota, are indicative of increased periodontitis lesions in adults (Hosaka et al., 1994; Umeda et al., 1996). Interestingly, it has been shown that the species found in this red complex have been found to produce proteolytic enzymes, a contributing factor to periodontitis disease progression (Loesche *et al.*,

1992), and have a strong capacity to co-aggregate (Grenier, 1992; Onagawa *et al*., 1994; Yao *et al*., 1996).

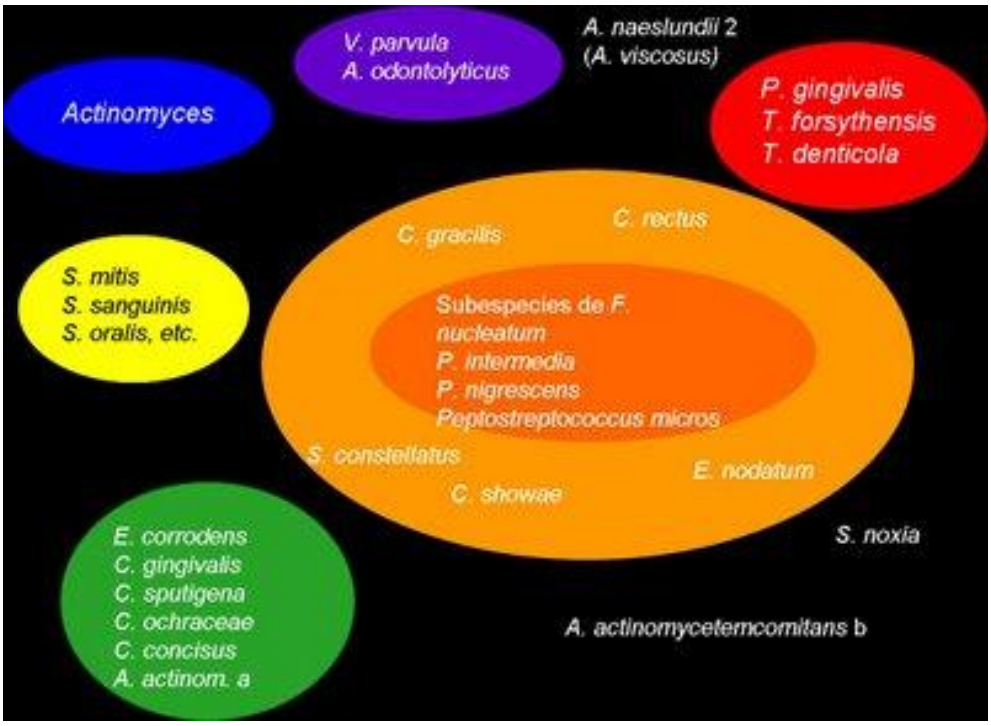


Figure 9 Diagrammatic representation of subgingival microbiota complexes (Socransky et al., 1998).

The 'orange complex' has also been strongly associated with conditions that affect the periodontal pocket, such as mobile teeth (Grant *et al*., 1992), periodontitis, (von Troil-Linden *et al.,* 1995), and infected root canals (Sundqvist, 1992). Investigations into bacteria associated with a healthy gingival crevice found that *Streptococcus* spp., *Actinomyces* spp., *Capnocytophaga* spp., *Eubacterium* spp., *Fusobacterium* spp. and *Veillonella* spp. predominate (Darveau *et al.*, 1997; Listgarten, 1994). Africa (2010) have shown a correlation between the prescence of organisms found in these orange and red complexes and an increased risk of pre-term delivery of low birth weight infants.

1.3.2.1 Gingivitis

Gingivitis is characterised by redness, swelling, bleeding gums on brushing or probing. The formation of plaque results in an inflammatory response in the gingiva, causing a characteristic redness and swelling. Swelling also results in increased leakage of gingival crevicular fluid, (GCF). GCF is an inflammatory exudate composed predominantly of serum, but also contains immunoglobulin G, (IgG), immunoglobulin M, (IgM), leukocytes, albumin and other cellular components, (Lamont *et al*., 2006). GCF washes the gingival crevice, helping to flush it free of bacteria and their metabolites many of which are toxic to the epithelial cells. In healthy gingiva GCF flow is low, but in patients with gingivitis it has been shown up to 40 µL can be recovered in as little as 15 minutes, (Lamont *et al*., 2006).

Gingivitis can develop within 10-21 days where oral hygiene regimes have stopped (Loe *et al.*, 1966). This can cause a shift to (Socransky *et al.*, 1998) predominantly gram-negative anaerobes and spirochetes. A number of bacterially-derived compounds can help elicit an innate immune response, including inflammatory mediators (Page, 1991). This causes local inflammation and swelling of the gingivae, which leads to the characteristic appearance of gingivitis (Bernimoulin, 2003). To the patient, gingivitis is characterised by bleeding gums when brushing (Pihlstrom *et al.*, 2005).

The keratinized epithelial covering of the upper and lower alveolar ridges is termed the gingiva and it surrounds and supports the teeth. In healthy individuals it is a pale pink colour. Gingivitis is a non-specific inflammatory response by the gingivae to the non-specific build-up of plaque. This condition is easily reversed by the removal of the built up plaque. It has been reported that gingivitis can affect between 50-90% of adults globally from time-to-time (Albander *et al.*, 2002).

Good oral hygiene procedures prevent the build-up and maturation of dental plaque and progression to gingivitis. A wide range of oral care products have been developed with the aim of disrupting the plaque biofilm and aiding in its dispersal. Many of these products contain antimicrobials in order to do this. The current gold standard antimicrobial for use in oral care formulations is chlorhexidine which is typically delivered as a mouthwash. However, to prevent gingivitis new actives should be sought that can be delivered a variety of routes to the oral cavity, e.g. toothpaste, gums.

1.3.2.2 Periodontitis

Periodontitis is more severe than gingivitis and results in the loss of both connective tissue and the surrounding bone, often leading to tooth loss. The leading cause of periodontitis is the deepening of gingival pockets due to inflammation brought on by the presence of undisturbed plaque. Interestingly, the disease is more prevalent in developing countries (van Palenstein Helderman *et*

al., 1996), with 22% of US adults reported to have mild disease, and 13% suffering more severe periodontitis (Albandar *et al.,* 1999).

Socransky and Haffajee (2003) highlight that the numbers of bacteria associated with supragingival plaque can exceed $1x10^9$ cfu, whereas a healthy gingival pocket typically contains $1x10^3$cfu. Although, it is generally accepted that various organisms within subgingival plaque are associated with the development and progression of periodontitis, including *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola* and *Aggregatibacter actinomycetemcomitans* [SIC] (Socransky & Haffajee,2003; Newman *et al.,* 1976), it is also becoming more evident that a number of key risk factors can aid in the development and progression of the disease, including several genetic conditions and environmental factors, such as smoking (Van Dyke and Sheilesh, 2005; Bergstrom, 2004; Michalowicz *et al.,* 1991). As recently discussed by Wade (2011), the identification of specific organisms associated with periodontitis can no longer be a key determination in treatment of the disease, rather the host immunological response to these organisms should be considered. This is key when we consider findings by Taubman et al., (2007) who found susceptible individuals would initiate excessive immune responses to normal microbiota in the oral cavity.

The prevention of periodontitis requires both the reduction and control of both bacteria and any associated risk factors. The application of a good oral care regime and guidance from a professional oral care expert has been shown by Axelsson *et al.,* (2004) to slow and stop periodontitis for many years, thus

reducing tooth loss. If, however, periodontitis is present, professional treatment is required, often including tissue debridement in the affected periodontal pockets. If supported by antibiotic therapy, either local or systemic, this often ensures a positive outcome for the patient (Haffajee *et al.,* 2003; Hung and Douglass, 2002).

1.3.3 Tongue Biofilms

The tongue surface is unique within the oral cavity due to its structure and large surface area. It has been proposed that the tongue is a reservoir for most salivary bacteria, including periodontal pathogens (Jacobson *et al.,* 1973; Lee *et al.*, 1999). The main species accredited with malodour formation within the oral cavity include *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Treponema denticola, Veillonella alcalescens, Tannerella forsythia, Peptostreptococcus* spp., *Eubacterium* spp., *Selenomonas* spp. and *Solobacterium moorei* (De Boever *et al.,* 1994; Greenman *et al.,* 2005; Hartley *et al.*, 1999; Kazor et al., 2003; Nakano *et al.*, 2002; Spratt and Pratten, 2003). These bacteria tend to reside in the crypts and fissures of the tongue (Greenman *et al.*, 2005; Nakano *et al.*, 2002) due to the low REDOX potential of this environment.

It has been estimated that 50% of the population could suffer from oral malodour (Grigor and Roberts, 1992), particularly after waking (Sanz *et al.*, 2001). However, only a small percentage of this population will suffer from clinical halitosis (Loesche and Kazor, 2002). The main components of oral malodour are considered to be the volatile sulfur compounds (VSC), which are chiefly comprised of hydrogen sulfide, methyl mercaptan and dimethyl disulfide (Kazor

et al., 2003). However, several other compounds add to the bouquet of oral malodour including putresine, cadaverine, indole, skatole, butyric and propionic acids (Quirynen, 2003; Spratt and Pratten, 2003).

The production of malodourous chemicals are commonly associated with bacterial metabolism (Kazor *et al.*, 2003; Koshimune *et al.*, 2003) and the putrefaction of proteins by anaerobic gram-negative organisms (De Boever *et al.*, 1994; Koshimune *et al.*, 2003; Quirynen, 2003; Quirynen *et al.*, 2004). Orally generated malodour can be controlled by a number of methods including masking, inactivation of malodorous molecules or antimicrobial agents (Carvalho *et al.*, 2004; Young *et al.*, 2003).

1.3.4 Oral Biofilms and Systemic Health

Recent research into periodontal disease is generating support for the hypothesis that there is an association between periodontal disease and systemic health, in particular, cardiovascular disease, adverse pregnancy outcomes, type-II diabetes and osteoporosis (Haraszthy *et al.*, 2000; Kim and Amar, 2006; Loos *et al*., 2000). This arena is researched greatly and debated widely as it is a relatively new research arena with most correlations not being fully elucidated. Interestingly, DNA from *Tannerella forsythia* (formerly *Bacteroides forsythus*), *F. nucleatum*, *P. intermedia*, *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) have been recovered from carotid atheromatous plaques by Cairo *et al.*, (2004). It would be easy to try and imagine a correlation between osteoporosis and periodontal disease in that there is

a shared clinical feature between both conditions of bone loss. However, little clinical correlation is available and any specific mechanism or association between both have not been fully elucidated. It should be noted that both may share key risk factors such as, inflammation, obesity and smoking, in the development and progression of each disease (Jeffcoat and Chestnut, 1993; Page *et al.,* 1985).

1.4 Methods for Oral Biofilm Analysis

1.4.1 Biofilm Visualisation Techniques

1.4.1.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is widely used as a visual aid where high resolution and a large field of view is required. This technique relies on the generation of electrons which excite surface electrons as the scanning beam moves across the sample in a square raster scan. Resolution of 30 Ångstroms (1 Ångstrom is equal to 0.1 nm or $1x10^{-10}$ metres) under optimal conditions is possible (EVO SEM, Carl Zeiss product literature). However, for biofilm structure the resolution is greatly reduced without initial fixation, staining, drying and metal sputter coating, which deposits a thin layer of either gold or platinum on the surface. Electrons can penetrate uncoated biological samples, giving an image of low resolution, however, if the samples are sputter coated, the electrons are reflected to the detectors, thus giving greater resolution of the surface topography (Kamper *et al.,* 2004). Although SEM can give good resolution, the high pressure required within the chamber causes dehydration and the generation

of artefacts (Fassel and Edmiston, Jr., 1999; Kamper *et al.*, 2004; Little *et al.*, 1991). The use of Environmental Scanning Electron Microscopy (ESEM) allows visualisation of biofilms within a damp atmosphere with moderate vacuum (Little *et al.*, 1991), allowing images to be taken on a biofilm surface without dehydration and the generation of artefacts. A number of investigators have utilised environmental scanning electron microscopy (ESEM) when imaging biofilms (Bergmans *et al.*, 2005; Priester *et al.*, 2006).

1.4.1.2 Atomic Force Microscopy

The Atomic Force Microscope (AFM) or Scanning Probe Microscope (SPM) was invented in 1986 by Binning *et al.* Like all SPM, the AFM utilises a sharp probe moving over the test surface of a sample in a raster scan. In the case of the AFM, the probe is on the end of a cantilever which bends in response to the force between the tip and the sample as in Figure 10. A laser beam deflects off the cantilever which is analysed by the AFM. Images are taken of the surface using two methods – contact (probe remains in contact with the surface during raster scan) and tapping (probe vibrates and taps the surface). Tapping mode is generally used for the study of biological samples where contact mode would merely push the material across the sample surface. AFM technology can have resolution as low as 10 Ångstroms (one full twist of DNA double helix = 34 Ångstroms (Mandelkern *et al*., 1981)).

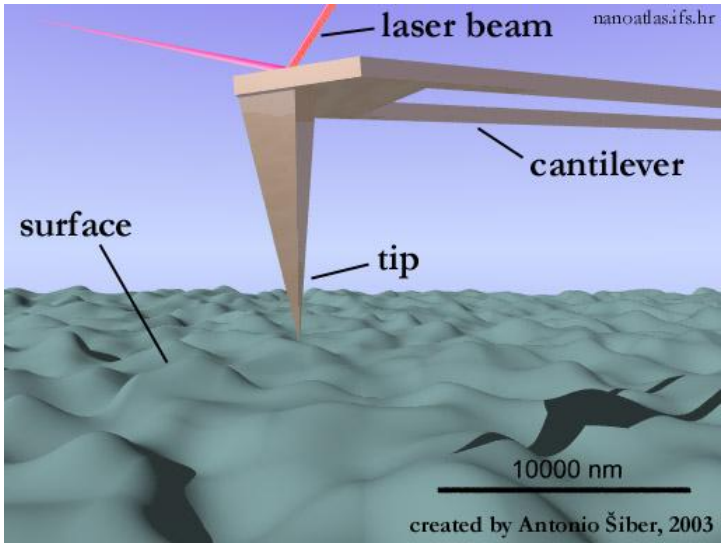


Figure 10. Schematic diagram of AFM cantilever moving over test surface.
Image taken from www.nanotech-now.com/Art_Gallery/antonio-siber.htm

A number of studies have utilised the AFM to investigate bacterial attachment (Postollec *et al.*, 2006; Whitehead *et al.*, 2006) and salivary pellicle (Berg *et al.*, 2003; Hannig *et al.*, 2004). Most analyses will collect height, amplitude and phase images. Where height images capture both the x/y- plane as well as the z-plane to give true results of the surface topography, however, visually it gives little clarity of the fine detail of the image. Another measurement, amplitude, often shows structures in finer details, as it shows a map of the slope of the surface; however, using this imaging technique renders the z-plane meaningless. Finally, phase images are an image of the interaction of the cantilever with the sample surface. With respect to the data obtained from height and amplitude images phase images can differentiate relative softness or hardness of the sample.

1.4.1.3 Confocal Microscopy

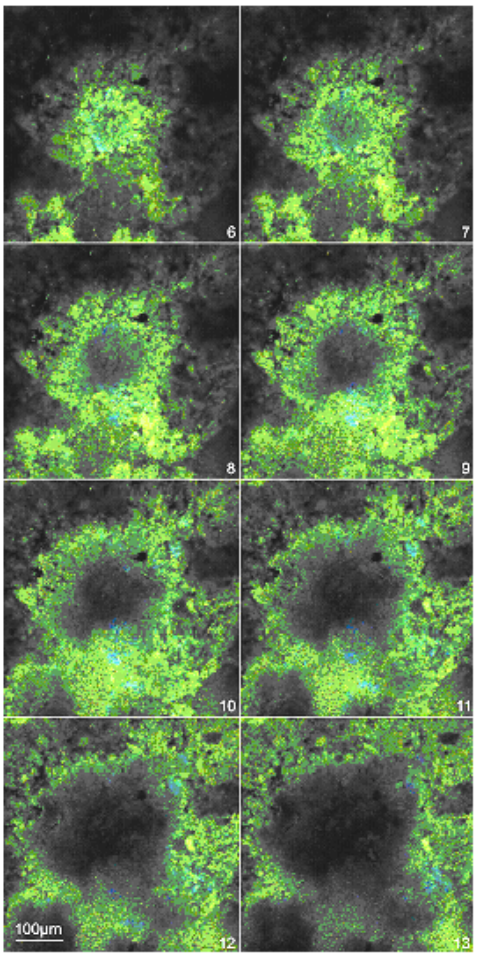


Figure 11 Optical sections (partial image stack) through a biofilm exhibiting the structural motif of an outer layer of viable bacteria. z-axis separation of optical sections = 2.9µm. Reproduced with kind permission from Dr. C. Hope. (Hope *et al.* 2003).

Confocal laser scanning microscopy (CLSM) permits optical sectioning of a specimen by capturing light from the local plane, thus CLSM with subsequent collation of the sectioning to produce a 3D rendering of the biofilm structure. An example of this sectioning can be seen in Figure 11. This method of biofilm visualisation is normally dependent on fluorescence, either derived from the specimen itself, or through staining. Research carried out by Caldwell (1992) utilised fluorescein isothiocyanate, staining the 'bulk' fluid or 'void volume',

leaving the biofilm biomass unstained; the biofilm was the 'negative' rendering of the image. Recently, SYTO stains (Invitrogen, Carlsbad, USA) have become available to stain specific organisms within the biofilm. Most commonly utilised stains are the LIVE/DEAD® staining where SYTO 9 stains all bacterial cells (independent of cell viability), whereas charged propidium iodide penetrates dead cells due membrane damage, thus allowing the stain to be internalised within the bacterial cell and when both dyes are present, reduce SYTO 9 to fluoresce red. This allows for differentiation of live and dead cells within the biofilm. However, this approach has been shown to be of limited use for environmental organisms (Shi *et al.*, 2007) due to changes in propidium iodide uptake during some environmental bacterial growth cycles. Visualisation of biofilms helps to understand structural differences and similarities that may be seen in comparison with those in vivo. Below is a summary of how models utilised to study biofilms can both provide insight into biofilms but can also distort what is seen clinically.

1.5 Oral Biofilm Models

The search for new antimicrobial agents for improved biofilm control within the oral cavity requires that appropriate screening models be put in place. Key criteria for these models would ideally include, the ability to be predictive of clinical outcome, the use of orally relevant organisms (mixed species), bacteria present in biofilms, short contact time relevant to the use of oral care products, reproducibility and high throughput.

1.5.1 Surfaces Used in Oral Biofilm Model Systems

To mimic the attachment of oral bacteria to saliva modified surfaces a number of substrata have been investigated including glass (Stinson *et al.,* 1981) and powdered enamel (Hillman *et al.,* 1970), to name a few. The following section discusses in more detail model substrata currently used with respect to this oral biofilm model system.

1.5.1.1. Hydroxyapatite

Hydroxyapatite has been used for over 40 years as a model for enamel dissolution studies (Higuchi *et al.,* 1965; 1969; Debhiya *et al.,* 1974). Gibbons (1976) looked at using hydroxyapatite coated in saliva as a substrate for the adherence of *Streptococcus miteor* and subsequent biofilm development. It has been shown that most bacterial adherence takes place within the first 10 minutes post-inoculation with saturation attachment observed after 30 minutes (Gaines *et al.,* 2003).

The problem with hydroxyapatite is its difficulty in adhering to the underlying substratum. Therefore, either hydroxyapatite discs or beads have been utilised. The author recommends using sintered hydroxyapatite as this reduces the porosity of the material which is generally manufactured by compression of powdered hydroxyapatite and then heated. However, performing adherence or biofilm assays on this material can be tedious due to the washing steps and can lead to physical disruption of the biofilm of interest. To address this, a number of methods have been developed to attach hydroxyapatite to a surface either via

deposition (Schilling *et al.*, 1994), or sputter-coating (Elliot *et al.*, 2005). Both allow the development of the biofilm with microtitre plates or flow cells, respectively. Using a contact angle goniometer the surface free energy of a hydroxyapatite sputter-coated surface has been shown to be 35.3m J m^{-2}, which is within the range shown to be representative of human tooth enamel (Christersson *et al.*, 1989).

1.5.2 *In situ* Model Systems

In situ oral biofilm models are considered the 'gold standard' of oral biofilm research as they permit the generation and testing of biofilms under their native environments, reflecting what would be seen clinically. A number of *in situ* models have been developed to investigate caries (Gameiro *et al.*, 2009; Zero, 1995), dentinal caries (Lima *et al.*, 2009), fluoride penetration through plaque (Robertson *et al.*, 1997; Watson *et al.*, 2004; Watson *et al*., 2005) and to monitor the effect of preservatives on oral biofilms (Arweiler *et al.*, 2008). These models can be split into two main groups, those in which the biofilm develops directly on to the subject's tooth, or models which rely on the development of biofilms on surfaces held within the mouth on either palatal devices or intra-oral splints. Within these structures, typically sterilised human enamel or dentine slabs, bovine enamel or hydroxyapatite discs can be inserted. These models however, are not utilised as screening models due to the small number of replicates permitted, but rather an important evaluation tool for allowing the research to be carried out in the environment of the mouth and exposed to all shear forces and salivary components.

1.5.3 *In vitro* Model Systems

1.5.3.1 Constant Depth Film Fermenter

The constant depth film fermenter (CDFF) concept was originally discussed in 1974 by Atkinson and Fowler, focussing primarily on a microbial film fermenter. Utilising this concept and building on research carried out by Coombe (1981), further model developments by Peters and Wimpenny in 1988, resulted in a model that would allow the development of replicate biofilms of a defined thickness. This model permits the development of biofilms between 50-600µm thick, due to the biofilms being grown in recessed pans. The thickness is limited by a static Teflon blade which sweeps excess biomaterial from the pans as the system rotates. This is a closed model system where the environmental gas, medium and inoculum can be controlled. The biofilms can then be easily removed and evaluated for bacterial counts, architecture and antimicrobial efficacy of any test substances. The CDFF model system has been widely used in oral biofilm development and research (McBain *et al.*, 2003; Metcalf *et al.*, 2006; Pratten *et al.*, 1998; Vroom *et al.*, 1999; Wilson, 1990).

1.5.3.2 Artificial Mouth

The artificial mouth model developed by Sissons *et al.* (1991), allows the long-term growth and development of dental plaque, derived either from clinical plaque or saliva samples. The methodology permits the addition of different nutrients and modification to the environment to understand their effects on the developing biomass. The biomass is grown on a Lux Thermanox cover-slip, where nutrients and test agents can be added via entry ports. Utilising this methodology Sissons

was able to show similarities in responses to changes in pH with addition of sucrose and urea, and mineralisation as measured by the deposition of Ca, PO_4 and F to that of natural dental plaques. As noted by the authors, although the system has been run for 6-weeks, after just 3-weeks, the biomass was too large for the cover-slip. A key feature of this 'artificial mouth' model is the capacity to monitor the metabolism and environmental changes within the biomass formed. This model has been widely used to evaluate caries development (Tang *et al.* 2003; Shu 1998; Shu *et al.* 2000) and viability (Filoche *et al.* 2007).

1.5.3.3 Microtitre Plate

Microtitre multi-well plates were identified for use in biofilm systems due to the high number of replicates and high throughput testing possible. The use of microtitre plates for determining the susceptibility and subsequent quantification of the antimicrobial activity of an agent against a developed biofilm has been investigated previously using a number of different microorganisms (Christensen *et al.*, 1985; Djordjevic *et al.*, 2002; Pitts *et al.*, 2003; Stepanovic *et al.*, 2000). Different studies have used a range of dyes and visual indicators, for example, crystal violet to stain attached organisms (Deighton and Balkau, 1990; Stepanovic *et al.*, 2000). This technique does not indicate the viability of the remaining biomass, however, other researchers have investigated the use of indicator dyes that can measure cellular metabolism such as, 1,5-cyano-2,3-ditolyl tetrazolium chloride and AlamarBlue™ (Baker and Tenover, 1996; Collins and Franzblau, 1997; Fields and Lancaster, 1993; Springer *et al.*, 1998). AlamarBlue™ (AB) can quantitatively measure the metabolism of bacteria and allows the investigation of

the antibacterial activity of test agents (Fields and Lancaster, 1993). The active component of AB has been identified as resazurin (O'Brien *et al.*, 2000). Under a reducing environment AB will elicit a change from the blue (unreduced) form to the pink (reduced) form. AB can be reduced by nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide ($FADH_2$), flavin mononucleotide ($FMNH_2$), nicotinamide adenine dinucleotide (NADH) and cytochromes (Biosource, product literature). It has therefore been proposed that the site of reduction is the electron transport chain (Springer *et al.*, 1998) reducing the blue resazurin to pink resorfin which also fluoresces (O'Brien *et al.*, 2000). AB has also been utilised as an indicator dye in planktonic bacterial susceptibility assays (Baker and Tenover, 1996; Collins and Franzblau, 1997) and is safe, reliable, sensitive and very simple to use.

Due to the hydrophobic surface of microtitre plates, a number of investigators have coated the wells with hydroxyapatite (HA) (Gaines *et al.*, 2003; Schilling *et al.*, 1994). In these studies, the HA was either precipitated on the surface (Schilling *et al.*, 1994) or formed into pellets for use (Gaines *et al.*, 2003).

1.5.3.4 Minimum Biofilm Eradication Concentration (MBEC) Model

The original model developed was called the Calgary Biofilm Device (CBD) and developed by Ceri et al., (1999). A key reason for utilising this methodology is that it is a peer reviewed, 96-well microtitre plate biofilm screening model. The MBEC model system utilises both the lid, which contains 96 pegs, corresponding to each of the 96-wells on the base of the plate. Once a test inoculum is added to

each of the wells on the microtitre plate and the plate incubated at an appropriate temperature and time, biofilms will form on the pegs. The model was developed to investigate the antibiotic susceptibility of attached bacteria.

This system has now been used in a number of studies including investigating the effect of metals on biofilms (Harrison *et al.*, 2005) and to evaluate antimicrobials for food and food contact surfaces (Ali *et al.*, 2006). The literature states that an average of $2x10^6$ cfu/peg for *Listeria innocua* (Harrison *et al.*, 2005), $5x10^7$ cfu/peg for *Pseudomonas aeruginosa* and $2x10^5$ cfu/peg with *S. aureus* (Ali *et al.*, 2006) are possible with this model. To evaluate further, the pegs can be broken and sonicated to determine minimum bactericidal concentration (MBC) and the spent medium in the 96-well plate can be analysed for minimum inhibitory concentration (MIC) (Harrison *et al.*, 2005). Although, Ali (2006) reported difficulties in aseptically removing the pegs for enumeration of attached organisms. The benefit offered by the CBD assay is the capability to test a range of antimicrobials with varied concentrations against reproducible biofilms in one assay. The model was not originally designed for use with oral organisms; therefore, concerns exist for the use of this model with these organisms. The problems arise from growing oral biofilms on non-orally relevant surfaces; such as polystyrene for the MBEC pegs. It has been shown that the chemical and physical properties of the underlying surface affects the development of the conditioning film including physiochemical surface properties, composition, density and configuration (Fletcher, 1976; Strevett and Chen, 2003). It has also been observed that oral bacteria can detach from a surface due to a cohesive fault

within the conditioning film (Busscher *et al.*, 1995). Parahitayawa (2006) discusses their findings of an increase in attachment of *Candida krusei* to the pegs of the MBEC in comparison with *C. albicans* attachment. Previous studies have shown that *C. krusei* is more hydrophobic than *C. albicans*, thus having a greater propensity to attach on these surfaces (Samaranayake *et al.*, 1995).

1.5.3.5 Sorbarod Biofilm Model System

Previous studies have demonstrated that the perfused Sorbarod biofilm system can maintain stable bacterial communities (Ledder *et al.*, 2006; McBain *et al*., 2005). Orally generated malodour can be controlled by a number of methods such as masking, inactivation/neutralisation of malodourous molecules or use of antimicrobial agents to kill the volatile sulphur compound (VSC) producing organisms (Carvalho *et al.*, 2004; Young *et al.*, 2003). A number of model systems exist for *in vitro* investigation of oral malodour including the CDFF (Pratten *et al.*, 2003b). The Sorbarod biofilm permits the development of an orally relevant mixed species biofilm model in which test actives can be evaluated for impact on VSC levels and their antibacterial ability under conditions of flow. The model is based on the Perfused Biofilm Model (Hodgson *et al.*, 1995), modified to include an orally relevant malodourous biofilm derived from tongue dorsum scrapings (Greenman *et al.*, 2005), and uses a Sorbarod filter (cylindrical paper sleeve encasing a compacted concertina of cellulose fibres, like a cigarette filter) to allow the attachment and growth of bacteria and continuous flow of media to provide a substantivity challenge when evaluating the efficacy of test agents. The model was used to compare the anti-VSC efficacy of various agents

with antimicrobial and/or VSC neutralising activity employing GC analysis for the determination of hydrogen sulfide and methyl mercaptan. Building on a model designed by Spencer *et al*. (2007) utilising a Halimeter to monitor total VSC's.

The main difference between the conditions used here and those reported by Greenman *et al.,* (2005) is the composition of the perfusion gas. Thus, the original Multiple Sorbarod Device model utilised anaerobic gas whereas the method reported here uses sterile lab air to better mimic the *in vivo* situation where the tongue and associated biofilm is exposed to air during breathing, speech etc.

1.6 Key Oral Care Actives Permitted in Oral Care Products

Many patients and consumers have difficulty in removing all plaque via mechamical means. Indeed, the increase in the incidence of fillings at the least accessiable areas, for example aproximal and occlusal fissures are testament to the inability to consistently removel built up plaque. Therefore, there is an increasing desire to supplement mechanical removal of plaque with an antimicrobial agent to help reduce and bacterial still remaining on the tooth's surface.

Currently there are a number of key antimicrobial agents that are permitted for use in oral care products, however for this PhD three were chosen to help test the developed biofilm model, as these actives have been widely studied previously and their activity was well understood.

1.6.1 Chlorhexidine

Chlorhexidine (1,1'-hexamethylene-bis-5(4-chlorophenyl bisguanide) is a broad spectrum, cationic antimicrobial. Due to its cationic nature, this antimicrobial is well studied for its ability to be retained on a biological surface and continue to provide antimicrobial effectiveness (Löe and Schiøtt, 1970; Mandell, 1994; Carrilho *et al*. 2010). This ability to attach to the surface is refered to substantivity and was first reported in 1971 (Rølla, G. *et al*. 1971). Chlorhexidine has often been referred to as the 'gold-standard' antimicrobial for use in the oral cavity, however, due to its inherent substantivity, chlorhexidine, can have negative properties. McRoy *et al.* (2008), reported taste changes, tooth staining, sore mouth and/or throat, and tongue irritation.

1.6.2 Triclosan

Triclosan (2,4,4'trichlor-2'-hydroxydiphenyl ether) is a chlorinated bisphenol antimicrobial. Its antimicrobial activity has been attributed to it's ability to inhibit the NADH or NADPH-dependent ACP reductase pathway, inhibiting bacterial fatty acid biosynthesis. Unlike chlorhexidine, triclosan has no substantivity to oral surfaces, however a number of researchers have investigated the use of polymers to increase triclosan substantivity, (Loftsson *et al*. 1999; Zaman *et al*. 2010; Steinberg *et al.* 2006) as this compound is not inherently substantive in the oral cavity.

Triclosan is widely used in the personal care category and this usage has been linked to the appearance of organisms containing efflux pumps for the removal of

triclosan (Yazdahankhah *et al.* 2006; Chuanchuen *et al*. 2003). Therefore, concerns exist about the continued used, for the generation of antimicrobial resistance to triclosan.

1.6.3 Zinc Salts

Zinc salts, more commonly, zinc gluconate, zinc chloride and zinc acetate have been used in oral care products for their ability to impart, abeit slight antimicrobial effect, but also its effect or oral malodour (Brunette *et al*. 1998; Young *et al.* 2001; Young *et al*. 2003). It is now generally accepted that oral malodour generates from the bacterial that reside in the crypts and fissures of the tongue. The odour is generated from the bacterial putrefaction of proteins found in the oral cavity (Kleinberg and Codipilly, 1997). Zinc salts have been shown in reduce oral malodour by chemical neutralisation of the main components of oral malodour, hydrogen sulphide and methyl mercaptan (Burnett *et al.*, 2011).

1.7 Aims and Objective of this Research

The overall aim of this research was the development of a reproducible 'off-the-shelf' assay to demonstrate the antimicrobial efficacy of test agents against orally relevant biofilms. It was envisaged that these biofilms will represent different microbiota associated with oral diseases including the tongue, supragingival and subgingival (in particular those responsible for gingivitis) populations. Therefore, the objective was to define each of the test parameters of the model to develop a robust and reproducible method for the evaluation of antimicrobials. The work focused upon media and incubation, inocula, surface attachment, biofilm structure and viability, as well as *in vitro* correlation.

The method outline was to grow orally relevant biofilms on a hydroxyapatite (HA) coated microtitre 96-well polystyrene plate. The intention of this approach was to fulfil as many of the required criteria as possible, i.e. orally relevant surface, relevant short contact time, rapid and reproducible with high number of replicates against a defined oral biofilm population such that a pharmaceutical company could have a high volume screening methodology for the investigations into novel test actives for use in oral care products.

CHAPTER 2: Materials and Methods

2.1 Bioplate Methodology

2.1.1 Manufacture of Hydroxyapatite Coated 96-Well Plates

Twenty grams of hydroxyapatite (HA) powder (Sigma-Aldrich, Gillingham, UK) was measured into a 115 mm diameter crystallising basin and placed on a magnetic stirrer. A magnetic stirrer bar was then added to the powder with 250 mL of acetone (Sigma, UK) and stirred so the powder was evenly suspended in the acetone. Each well of a 96-well microtitre polystyrene plate (VWR, Lutterworth, UK) then received 60 µL of the suspension; the plate was placed on an orbital shaker set at 200 rpm in a 30°C incubator. After drying, loose HA powder was removed by inverting and tapping sharply. Plates were subsequently washed in dH_2O to further remove any remaining loose HA and allowed to dry at 37°C overnight. Any plate showing poor coverage (visible cracks or bubbles in the HA layer by eye) were removed and discarded. Plates were then sterilised by placing in a UV cabinet overnight (Steristrom, DaRo, Sudbury, UK) at 254 nm.

2.1.2 Preparation of Bioplates

Full testing methodology concerning the donation, sampling, incubation and testing of the organisms used in this model is summarised here. The full testing methodology can be found in section 2.2. Two separate aliquots of 250 mL of tryptone soya broth (TSB; Oxoid, Basingstoke, UK) were inoculated with 3 mL of fresh human saliva pooled from three individuals who had abstained from any oral hygiene regime for 8 hours prior to collection. All donated saliva followed current GlaxoSmithKline protocols. One flask was incubated anaerobically

without shaking, the other incubated aerobically with shaking, both for 5 hours at 37°C. The contents of both flasks were combined and swirled gently to mix. Each well of a HA coated 96-well microtitre plate was inoculated with 20 µL of the freshly prepared bacterial culture. Subsequently, 180 µL of sterile TSB was added to each well, plates were then incubated aerobically for 17 hours at 37°C on an orbital shaker set at 200 rpm.

When analysing the antimicrobial activity of test agents, the plates containing the test agents were prepared in advance. To each column on an uncoated 96-well microtitre plate was added 200 µL of the test agent. This generated 8 replicates in one column for each test agents and permitted the evaluation of 10 actives with deionised water and 0.2% v/v chlorhexidine (CHX, 20% v/v CHX digluconate stock, (Sigma, UK)) used as negative and positive controls, respectively. Taking an inoculated Bioplate that had been incubated overnight, exhausted media from each well was removed using a Nunc-Immunowash 12 (Nunc, Kamstrup, Denmark) manual plate washer fed with TSB and attached to an effluent bottle via a vacuum pump. Wells were washed (x2) using the Immunowash to remove unattached or loosely bound bacterial cells, thus leaving the wells of the Bioplate empty. Using a 96-Transtar pipette (Corning, Sigma-Aldrich) and the uncoated 96-well plate, 200 µL of the test agents were transferred to the now empty Bioplate and incubated at 37°C for 2 minutes while shaking. Subsequently, using the Nunc-Immunowash, each well was washed (x2) with sterile TSB to remove traces of the test agent leaving the Bioplate empty. Using an 8x multichannel pipette, 200 µL of sterile TSB was added to each well on the plate and re-

incubated for 4 hours at 37°C. To measure the viability of the biofilm, 10% v/v solution of AlamarBlue™ (Serotec, Oxford, UK) in TSB was prepared. After the second incubation, the Bioplate was removed from the incubator and each well washed twice with sterile TSB. Using an 8x multichannel pipette, 200 µL of the 10% v/v AlamarBlue™ solution was added to each well. The plate was further incubated for 30 minutes at 37°C with shaking; Figure 12 shows a CHX dose response using AlamarBlue. Fluorescence was measured using a microtitre plate reader (Biotek, VT, USA) using a 530 nm excitation filter and a 590 nm emission filter. Results were recorded as mean fluorescence units (MFU) where n=8.

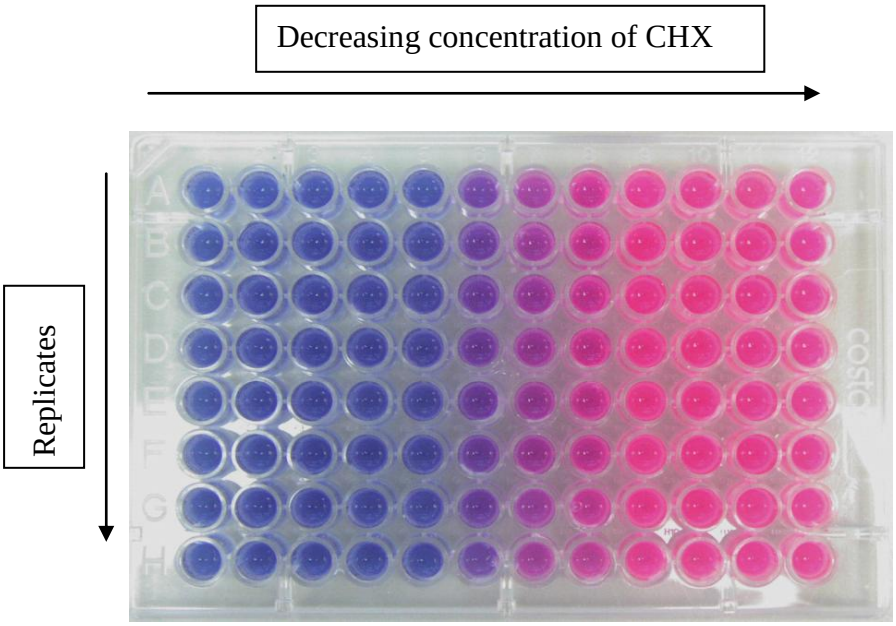


Figure 12. Image of CHX dose response in Bioplate with 10 %v/v AlamarBlue™. Pink shows increase in bacterial metabolism, blue shows a decrease in bacterial metabolism

2.2 Organisms Used to Develop Biofilms on HA Coated Plates

2.2.1 Mixed Species Salivary Samples

Initial research carried out utilised donated saliva with the aim to replicate mixed consortia that would be representative of the oral cavity. Consent was received for all donated saliva and samples taken from 3 individuals working at GlaxoSmithKline, Weybridge, UK. Unstimulated samples were donated in the morning prior to any oral care regime, with no eating permitted prior to donation. Subjects were requested to donate a minimum of 5 mL of saliva into 30 mL sterilin sterile containers (VWR), these were subsequently refrigerated (<2hours) until required. All saliva was combined to ensure a more representative oral microbiota of what would be found in the oral cavity. Three millilitres of the pooled saliva was added to each of two 250 mL brain heart infusion (BHI) broths (Oxoid) and incubated static at 37°C anaerobically, the other was incubated at 37°C with shaking aerobically for 5 hours. Subsequently, they were mixed together 20 µL was then added to each well of a hydroxyapatite coated 96-well microtitre plate.

2.2.2 Defined Species Inoculum

For a number of reasons, ease of use, reproducibility and safety, it was desirable to standardise the model inocula, as well as increasing opportunity of use, due to the introduction of the Human Tissue Act, 2004. This part-time PhD was initiated at the beginning of 2004 and therefore the HTA would have impacted the use of human derived salivary inocula. A move towards a defined salivary inoculum

was investigated. These organisms detailed below were specific oral bacteria chosen based on the paper "The establishment of reproducible, complex communities of oral bacteria in the chemostat using defined inocula." McKee *et al*. (1985). The organisms used in this paper were (reported as in the paper): *Streptococcus mutans* ATCC 2-27351, *Streptococcus sanguis* NCTC 7865, *Streptococcus mitior* EF 186, *Actinomyces viscosus* WVU 627, *Lactobacillus casei* AC 413, *Neisseria sp*. A 1078, *Veillonella alkalescens* ATCC 17745, *Bacteroides intermedius* T 588, and *Fusobacterium nucleatum* NCTC 10953. Due to the unavailability of some of these organisms, they were replaced with representative NCTC strains. The final list of organisms chosen were, *Streptococcus mutans* NCTC 10449, *Streptococcus sanguinis* NCTC 10904, *Streptococcus mitis* NCTC 12261, *Actinomyces viscosus* NCTC 10951, *Lactobacillus casei* NCTC 10302, *Neisseria mucosa* var. *mucosa* NCTC 10774, *Veillonella dispar* NCTC 11831, *Prevotella melaninogenica* NCTC 11321 and *Fusobacterium nucleatum* NCTC 10562. These organisms were grown up on either tryptone soya agar (TSA) or blood agar (BA) with 6% defibrinated horse blood (both Oxoid). Following the manufacturer's instructions for breaking the glass seals, each was resuspended in 1 mL of BHI broth and aspirated via a pipette. One hundred microlitres of this was plated out on appropriate media, aerobic organisms were incubated at 37°C for 48 hours. Anaerobic organisms were incubated for 7-10 days. Post incubation, representative colonies were removed and placed on agar slopes as stock organisms and placed in 4°C refrigeration for 1 month.

2.3 Visualisation of Biofilms

2.3.1 Scanning Electron Microscopy (SEM)

Twenty microlitres of a 10^8 cfu/mL stock solution of *S. mutans* NCTC 10449 was added to 180 µL of either TSB or BHI in a microtitre plate and incubated at 37°C for 24 hours aerobically with shaking (TSB) and without (BHI) shaking. Subsequently, the wells were washed using the method described previously and left to dry. A 6 mm borer (B&Q, UK) was used to remove a section from the base of the Bioplate well. Microscopic analysis of uninoculated HA coated wells and biofilms was then carried out using a Zeiss EVO scanning electron microscope operated at 2.5-3 kV.

Figure 13 Multi-stub holder which would allow investigation of more than one sample at a time in SEM.

Samples were mounted onto standard SEM stubs (Agar Scientific, Stansted, UK), as in Figure 13, with double sided carbon sticky dots (Agar Scientific). Samples were uncoated and representative images of the native HA coated surface were also taken.

2.3.2 Atomic Force Microscopy (AFM)

S. mutans NCTC 10449 was inoculated into 2 x 20 mL BHI broth and incubated at 37°C overnight with shaking at 200 rpm. A glass coverslip was added to one of the containers to allow attachment of the bacteria to the surface. A 10 µL aliquot was removed from the other container and placed onto a sterilised glass cover slip and allowed to dry. Glass coverslips were used in this analysis as the surface topography of hydroxyapatite coated polystyrene exceeded the z-plane allowed by the AFM.

Analysis was carried out using a Multimode V AFM (Vecco, California, USA), using a NP-20 silicon nitride probe with a tip radius of 20 nm (Veeco Instruments Ltd, Cambridge, UK) as in Figure 14.

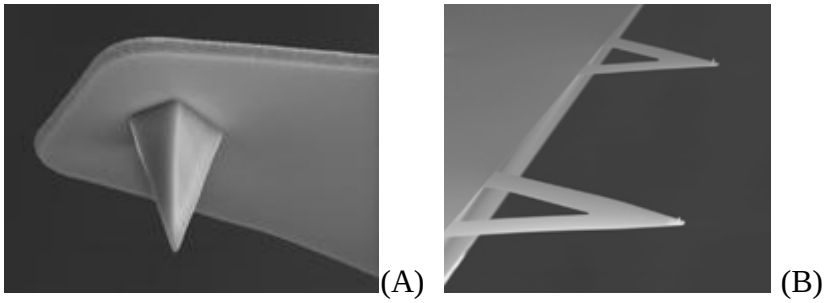

(A) (B)

Figure 14 Image of NP-10 silicon nitride probes which are representative of NP-20 probes, showing tip (A) and cantilever (B). Both images from Veeco website www.veeco.com

The AFM was operated in tapping mode (as contact mode would merely push the organisms) with images being taken at a speed of 0.447 – 0.671 Hz, with scan size ranging from 6.71 – 12.5 µm, line direction in all samples was retrace. Each image was recorded for height, amplitude and phase.

2.3.3 Confocal Laser Scanning Microscopy (CLSM)

CLSM was used in conjunction with LIVE/DEAD BacLight™ bacterial viability kit (Invitrogen, Paisley, UK) utilising two fluorescent dyes. The two dyes are reliant on the permeability of a cell surface to differentiate those which are membrane-compromised and those that have an intact membrane structure. STYO® 9 will label live and dead bacteria with green fluorescence, however, when used in conjunction with propidium iodide, which is more permeable, and better able to transverse cell membranes, dead cells with permeable membranes will fluoresce red. Red/green ratio can then be used to correlate viability of the sample.

Biofilms were removed from either the CDFF, MBEC or Bioplate and placed in a small cell-culture dish (Bibby Sterilin Ltd, UK) with 10 mL distilled water containing 2 µL of the LIVE/DEAD BacLight™ bacterial viability stain. These were incubated in the dark to allow the stain to develop for 15 minutes and rinsed to remove excess stain. The biofilm structure was examined with an Olympus BX51 microscope to which a Bio-Rad Radiance 2100 laser scanning system and a LUMPlanFI ×40 water lens was incorporated. Two-channel (viable "Live" and nonviable "Dead") confocal image stacks were collected in eight-bit colour depth at a resolution of 1024 x 1024 pixels. The *z*-axis step size was optimized for each image stack, depending upon the total depth of each of the test samples. Images where collated and analysed using Image J software, available at http://rsbweb.nih.gov/ij/.

2.4 Minimum Biofilm Eradication Concentration (MBEC) Model

The original model developed was called the Calgary Biofilm Device (CDB) was developed by Ceri *et al*., (1999). A key reason for utilising this methodology is that it is a peer reviewed, 96-well microtitre plate biofilm screening model. The MBEC model system utilises both the lid, which contains 96 pegs, corresponding to each of the 96-wells on the base of the plate, see Figure 15b. Once test inoculum was added to each of the wells on the microtitre plate and the plate incubated at an appropriate temperature and time, biofilms formed on the pegs as in Figure 15a. The model was developed to investigate the antibiotic susceptibility of attached bacteria.

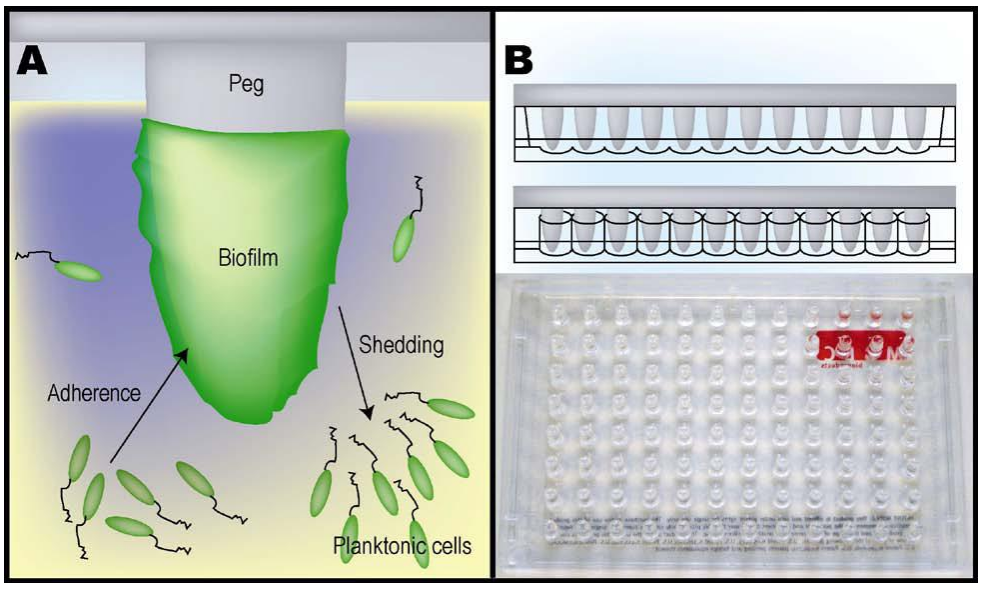


Figure 15 The MBEC™ High-throughput (HTP) Assay. A) Biofilms form on the polystyrene pegs B) The peg lid has 96 identical plastic pegs. Taken from www.innovotech.ca/documents/MBECHTPInstructions_Rev2.pdf

A summary schematic of the methodology can be found in Figure 16.

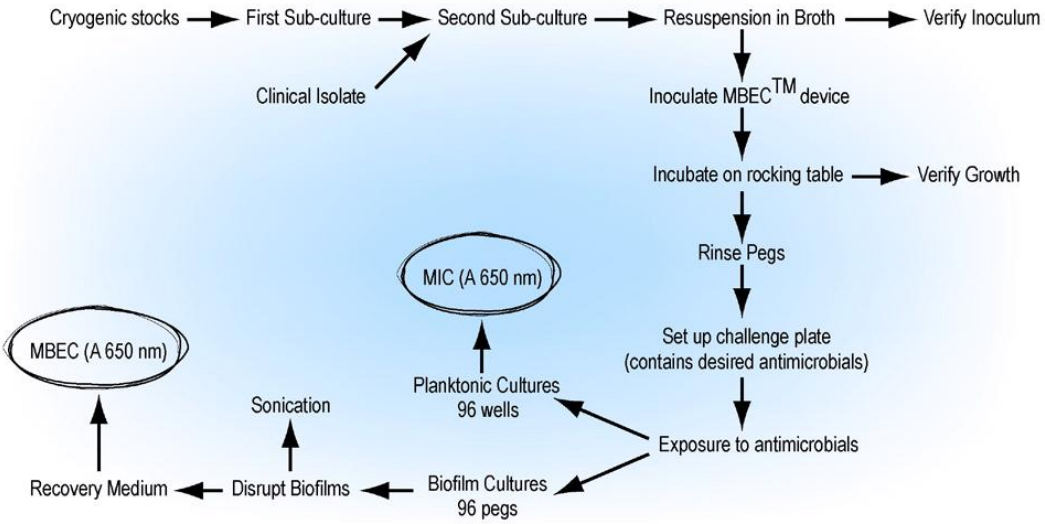


Figure 16 Overview of the MBEC testing methodology, taken from Innovotech methodology (http://www.innovotech.ca/products_use.php)

2.4.1 Antimicrobial Challenge for MBEC

A number of different antimicrobial challenges were carried out, described below are details carried out of a dose response of a chlorhexidine (CHX). Further testing can be found in chapter 5. Using a new microtitre plate, sterile deionised water was added to column 1 as a standard control and from column 2-9 was added varying concentrations of CHX including 2, 1, 0.5, 0.25, 0.1, 0.05, 0.01, 0.001% v/v, respectively.

After removing the inoculated MBEC plates from the incubator, the plates were rinsed in a separate plate, prepared previously, which contained R+P buffer solution. Washing was carried out to remove any unattached or loosely bound organisms. Three pegs were removed from column 12 using sterile forceps and each transferred to 10 ml of R+P diluent. This was subsequently vortexed for 1

minute and serially diluted from 10^{-1} to 10^{-7} and plated in duplicate on appropriate agar, then incubated with respect to the requirements of the individual organism. The remaining pegs, still attached to the lid, were transferred to the 96-well microtitre plates containing the CHX and placed in 37°C incubator for two minutes. Subsequently, the plate was removed from the incubator and rinsed in a separate plate, prepared previously, which contained R+P buffer solution. Two hundred microlitres of 10% AlamarBlue™ in BHI was added to a separate 96-well microtitre plate. The washed pegs were then added to this and re-incubated for 30 minutes at 37°C with shaking. Fluorescence was measured using a microtitre plate reader (Biotek) using a 530 nm excitation filter and a 590 nm emission filter. Results are recorded as mean fluorescence units (MFU).

2.5 Constant Depth Film Fermenter (CDFF)

The CDFF employed in this study was a modified version first developed by Peters and Wimpenny in 1988. The model was manufactured by John Parry-Jones Engineering (Cardiff, U.K) (Figure 17). The methodology utilised is based on that published by Pratten (2007).

Figure 17 Constant depth film fermenter with power unit and turntable (Pratten, 2007).

2.5.1 Assembly of the Constant Depth Film Fermenter

The CDFF has been well reported and below is the methodology employed. The CDFF contains a stainless steel turntable which houses up to fifteen polytetrafluoroethylene (PTFE) pans. Each pan contains up to five, 5 mm diameter holes which contain removable and adjustable PTFE plugs. Biofilms can be grown directly on to these plugs, or a substrate of interest can be added. In the present thesis hydroxyapatite sintered discs (5 ± 0.2 mm diameter, 1.8 ± 0.2 mm deep, Clarkson Chromatography, South Williamsport, USA) were utilised. As the turntable rotates at 3 revolutions per minute, it passes by two angled scraper blades which remove any excess biomass, keeping the biofilms at a constant depth. In the middle of each pan is a threaded hole for ease of removal of the unit from the CDFF. The removal of individual pans results in up to 5

replicate biofilms. These biofilms can them be utilised for further investigations such as culture plating or CLSM.

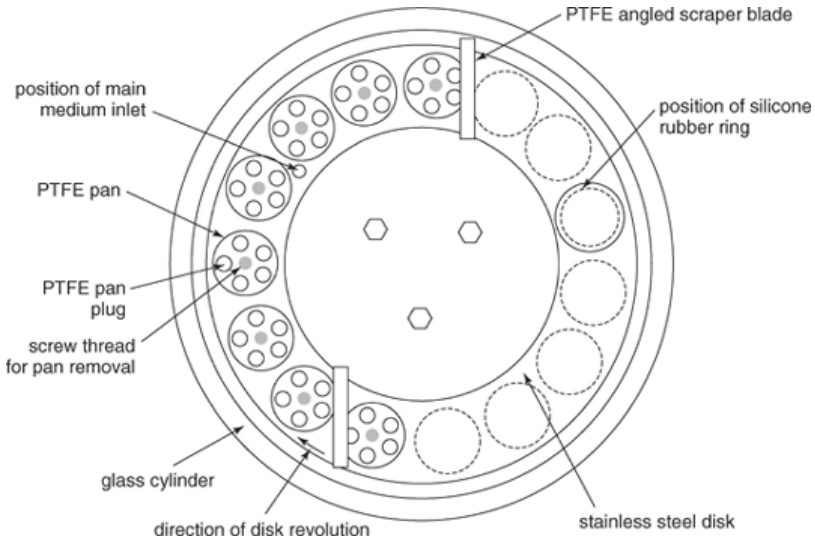


Figure 18 Sectional schematic showing the layout of the PTFE pans with scraper blade (Pratten, 2007).

The turntable unit was held inside a borosilicate glass unit held between two stainless stain plates with in/outlet ports, sealed using PTFE seals and high vacuum silicon grease (Dow Corning, Midland, MI, USA). All air filter ports were sealed using Whatman Hepa-vent air filters (0.2 µm) (VWR). All tubing was silicone based (Saint Gobain, Charny, France) and sealed using quick-connect fittings (VWR). The CDFF unit was sterilised via autoclaving at 121°C for 15 minutes.

2.5.2 Inoculation of the Constant Depth Film Fermenter

Full details of the inoculation of the CDFF can be found in chapter 5. A brief overview is given below. A defined inoculum was used to inoculate the CDFF, and was plated out on relevant media (full details can be found in Chapter 4) to obtain required concentrations. The organisms used were, *S. mutans* NCTC 10449 ($1x10^8$ cfu/mL), *S. mitis* NCTC 12261 ($1x10^{10}$ cfu/mL), *S. sanguinis* NCTC 10904 ($1x10^6$ cfu/mL), *A. viscosus* NCTC 10951 ($1x10^6$ cfu/mL), *L. casei* var. *rhamnosus* NCTC 10302 ($1x10^{10}$ cfu/mL), *N. mucosa* NCTC 10774 ($1x10^9$ cfu/mL), *V. dispar* NCTC 11831 ($1x10^5$), *P. melaninogenica* NCTC 11321 ($1x10^9$ cfu/mL) and *F. nucleatum* NCTC 10562 ($1x10^5$ cfu/mL). One millilitre of each organism; *S. mutans, S. mitis, S. sanguinis* and *L. casei* was added along with 4 mL of *A. viscosus, N. mucosa, V. dispar, P. melaninogenica, F. nucleatum* to 300 mL of artificial saliva.

The inoculum was added to the CDFF for 5 hours at a rate of 0.5 mL/min, incubated at 37°C aerobically and continually mixed with a magnetic stirrer. This was equivalent to the mean salivary flow rate of 0.72 litres/day for humans (Guyton, 1992). Subsequently, the inoculum was removed and the CDFF was then continually fed with artificial saliva at a rate of 0.34 mL/min. The CDFF unit was incubated at 37°C aerobically. At various time points biofilms were removed for analysis.

2.5.3 Operating Conditions of the Constant Depth Film Fermenter

The CDFF was held in the incubator at 37°C aerobically, with an artificial saliva feed at a rate of 0.34 mL/min. Effluent was collected down-stream from the CDFF, as seen in Figure 19.

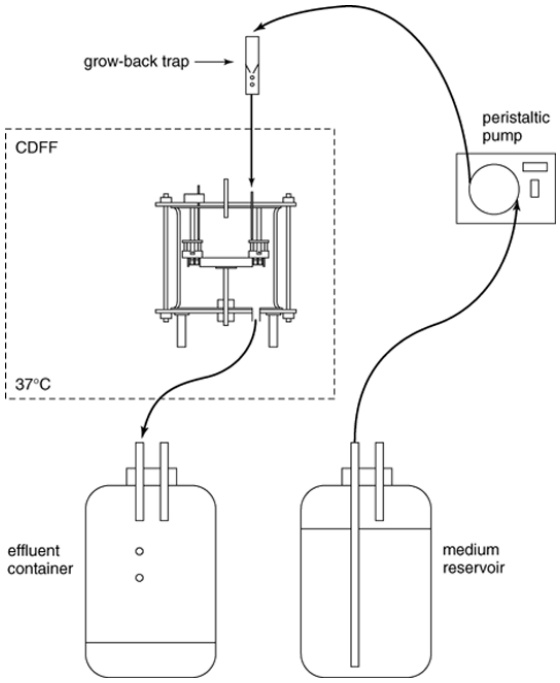


Figure 19 Schematic of the CDFF when running continuously – the dotted line represents the incubator (Pratten, 2007)

2.5.4 Sample Removal of the Constant Depth Film Fermenter

At defined time intervals, the CDFF rotation was stopped, allowing the removal of the biofilms grown on the PTFE pans to be removed via the sampling port. Sterilisation of the sampling tool and sampling port was carried out with 70% v/v ethanol. Once removed the pans were placed in diluent awaiting analysis and held for maximum 4 hours.

2.6 Sorbarod Biofilm Model

2.6.1 Assembly of Model

A schematic diagram of the Sorbarod biofilm system is shown in Figure 20, three of which were run in parallel.

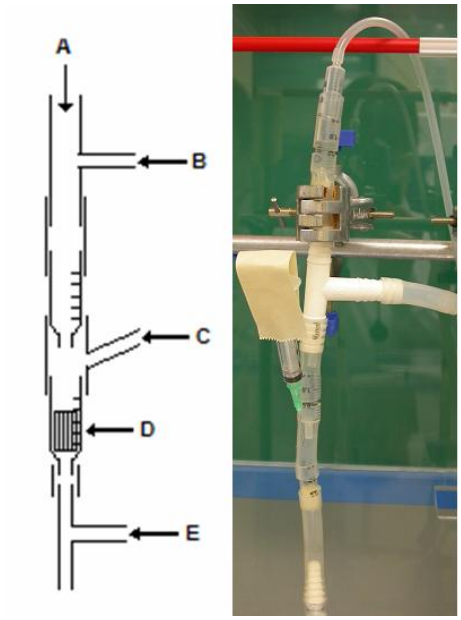


Figure 20 Schematic and photograph of Sorbarod Biofilm Model system A) media inlet 36 mL h^{-1} B) gas inlet by peristaltic additions 50 mL h^{-1} C) test agent addition via syringe D) biofilm containing Sorbarod filter E) gas sampling outlet.

Disposable 2 mL plastic syringes were cut and orientated using silicon tubing (Ø 1 cm, VWR, Lutterworth, UK). Medium was perfused through the Sorbarod (Figure 20A) at a flow rate of 36 mL/hour using a multichannel peristaltic pump (Watson and Marlow, Falmouth, UK). Sterile lab air was introduced via a side arm (Figure 20B) at a flow rate of 50 mL/hour using a single peristaltic pump (Watson and Marlow). A syringe was placed above the Sorbarod filter to allow introduction of the test agent. Downstream from the Sorbarod filter was a three-way tap leading to a 0.45 µm air filter (Pall Gelman, VWR) with 4 cm attached

silicon tubing. A gas-tight 20 mL syringe could be connected to the outlet to allow sampling of gas for analysis. Effluent from each Sorbarod flowed to a 2L waste carboy.

2.6.2 Preparation of Inoculum

The inoculum was generated using a sterile toothbrush (Macleans, GSK, Brentford, UK), autoclaved at 121°C for 15 mins, firmly brushed against the posterior dorsum of the tongue of a single donor and then washed in 10 mL of sterile phosphate buffered saline (PBS) to remove tongue biofilm. These steps were repeated until an optical density equivalent to a 3.0 McFarlane standard was obtained. The bacterial suspension in PBS was then added to 10 mL of sterile double strength Brain Heart Infusion (BHI) broth (Oxoid). The inoculum (2 mL) was added to a sterile Sorbarod housed in a 2 mL syringe (Figure 20C) in the absence of flow. Once inoculated, Sorbarod filters were incubated overnight in an anaerobic cabinet at 37°C, then connected to the gas, medium and effluent lines, and incubated for a further 48 h with continuous perfusion of gas and medium. Previous experiments showed that steady state VSC levels were reached after 48 hours incubation of the inoculated Sorbarod under the conditions reported here (data not shown).

CHAPTER 3: Methodology refinement of an *in vitro* hydroxyapatite microtitre biofilm model.

3.1 Introduction

Many *in vitro* oral biofilm studies require the development of orally relevant plaque. However, many studies focus primarily on single-species biofilms which do not take into consideration the multi-species interactions which occur in plaque (Adams *et al*., 2002; Luppens *et al*., 2008; Palmer *et al*., 2001). *In vitro* biofilm structure is determined by available nutrients, substratum in the model, organisms present and incubation conditions. Changes in any one of these parameters will affect bacterial growth and biofilm development, thus affecting biofilm structure. Biofilms have also been shown to be more resistant when under an antimicrobial challenge compared with planktonic cultures of the same organism (Costerton *et al*., 1995; Larsen and Fiehn, 1996; Wilson, 1996). This has shown to be as much as 1000-fold increase in resistance (Mah and O'Toole, 2001).

The development and understanding of well characterised high-throughput laboratory model is essential for mass screening of possible anti-plaque agents and formulations. The methodology should not only allow the screening of different test agents, but allow the evaluation and optimisation of test agent activity for example, by changing pH or addition of exipients to increase activity.

The effect of different surfaces on these models should be considered when it is known that the chemical and physical properties of the underlying substratum can affect the development of an acquired pellicle including its composition, density and configuration. These changes in the physico-chemical properties of the

acquired pellicle may influence bacterial attachment and subsequent biofilm development.

A number of different surfaces have been used in oral biofilm model development including glass (Stinson *et al.,* 1981), powdered enamel (Hillman *et al.,* 1970), cellulose (Greenman *et al.,* 2005), membrane filter and agar (Milward and Wilson, 1989) and polystyrene (Ceri *et al.,* 1999) to name a few. Hydroxyapatite has been used for over 50 years as a replacement for enamel (Higuchi *et al*., 1965; 1969). Using a Contact Angle Goniometer the surface free energy of a hydroxyapatite sputter-coated surface was 35.3m J m^{-2}, which is within the range shown to be representative of human tooth enamel (Christersson *et al.,* 1989). The problem with hydroxyapatite is its difficulty in adhering to the underlying substratum in the model. Therefore, either hydroxyapatite discs or beads have been previously utilised. However, performing adherence or biofilm assays on this material can be tedious due to the washing steps and can lead to physical disruption of the biofilm of interest or the disruption of the hydroxyapatite from the substratum.

When developing an orally relevant biofilm model, the closer we can be to the *in situ* scenario, the more representative of the *in vivo* effects can be investigated *in vitro.* Hence, to evaluate the efficacy of antimicrobial test agents, firstly, relevant biofilms should be employed and, secondly, growing on representative surfaces found in the oral cavity to determine active concentrations that may eliminate the organisms growing within these biofilm structures. The initial aim of this

research was the development of an assay to demonstrate the antimicrobial efficacy of test agents against orally relevant biofilms permitting high through-put test agent screening of possible anti-plaque agents, or indeed formulations.

3.2 Materials and Methods

3.2.1 Hydroxyapatite Surface for Biofilm Development

The development of a 96-well hydroxyapatite (HA) containing biofilm model required understanding of the associations between the bacteria and the surface. This experiment aimed to understand surface differences between HA discs and HA coated wells. AFM studies were carried out to show differences between planktonic organisms settled under the force of gravity to the bottom of the wells and that of bacteria which had attached and were in the very early stages of biofilm formation. This is important as using a system such as this where there is little or no shear, it is necessary to consider whether the organisms are actively attaching to the surface, or are being deposited. Initial work investigated how to differentiate between attached and planktonic organisms, by direct comparison with imaging from the AFM.

3.2.1.1 Hydroxyapatite Discs

Dense HA discs (5 mm diameter, 1.8 mm deep (Clarkson Chromatography Products, South Williamsport, USA)) were autoclaved at 121°C for 15 minutes. Using sterile forceps, one disc was transferred to each of the wells of an uncoated polystyrene 96-well plate. To this, $1x10^6$ cfu/mL of *S. mutans* in 180 µl of test

medium was added into each well of a 96-well plate. Each plate was incubated as appropriate; *S. mutans* was incubated at 37°C for 24 hours aerobically on an orbital shaker set at approximately 200 rpm. After incubation, the HA disc was removed using sterile forceps taking care not to touch the top surface. The disc was gently washed in R+P to remove any unattached bacteria and remove residues of the test medium.

3.2.1.2 Hydroxyapatite Coated Surface

Twenty µL of a 10^8 cfu/mL stock solution of *S. mutans* NCTC 10449 was added to 180 µL of BHI in a microtire plate and incubated at 37°C for 24 hours aerobically without shaking to allow attachment and biofilm development. Subsequently, the wells were washed using the method described in chapter 2 and left to dry. A 6 mm borer (B&Q, UK) was used to remove a section from the base of the well. The analysis of uninoculated HA coated wells and biofilms was carried out using a Zeiss EVO scanning electron microscope operated at 2.5-3 kV. Samples were mounted onto standard SEM stubs (Agar Scientific, Stansted, UK) with double sided carbon sticky dots (Agar Scientific). Samples were uncoated and representative images of the surface were taken.

3.2.1.3 Scanning Electron Microscopy

Samples of hydroxyapatite coated wells and dense hydroxyapatite discs (which were exposed to bacteria) were imaged. Test samples were mounted on appropriate stubbie holders (Agar Scientific, Standsted, UK) and imaged uncoated

in a Zeiss EVO Scanning electron Microscope operated at 3.5-3.75 kV (Carl Zeiss SMT Ltd, Cambridge, UK).

3.2.1.4 Atomic Force Microscopy

S. mutans NCTC 10449 was inoculated into 2 x 20 mL BHI broth with shaking to prevent settling and biofilm development of the base of the container, and incubated at 37°C overnight. To one container was added a glass coverslip to allow attachment of the bacteria to the surface. From the non-coverslip containing inoculum, a 10 µL aliquot was removed and placed onto a sterilised glass cover slip and allowed to dry. Glass coverslips were used in this analysis as the surface topography of hydroxyapatite coated polystyrene exceeded the z-plane allowed by the AFM. Analysis was carried out using a Multimode V AFM (Vecco), using a NP-20 silicon nitride probe with a tip radius of 20 nm (Vecco). The AFM was operated in tapping mode with images being taken at a speed 0.447 – 0.671 Hz, with scan size ranging from 6.71 – 12.5 µm, line direction in all samples is retrace.

3.2.2 Correlation Between Saliva Inocula and Bioplate Recovery

To investigate the correlation between saliva inoculum and Bioplate recovery, a microtitre plate biofilm disruption method was developed to optimise bacterial cell recovery. This methodology was utilised throughout this research.

3.2.2.1Biofilm Disruption

Due to the nature of biofilm growth (micro/macro colonies associated with a surface) it is feasible that when determining bacterial viability all of the organisms may not be removed from the surface and that aggregates of bacteria may form and give inaccurate counts. Therefore, the aim of this experiment was to develop a reproducible method for the disruption of biofilms grown on hydroxyapatite coated surface and dense hydroxyapatite discs. The protocol developed aimed to ensure bacterial recovery was an accurate presentation of actual bacteria present; therefore the method used only single species at a time.

3.2.2.2 Preparation of HA Discs and HA Coated Bioplates

S. sanguinis NCTC 10904 was grown on tryptone soya agar (TSA) (Oxoid, UK) at 37°C for 24 hours and *V. dispar* NCTC 11831 was grown on brain heart infusion agar (BHI) (Oxoid) agar at 37°C for 48-120 hours as orally representative organisms in this method development. Suspensions of 10^8 cfu/mL were prepared in reduced transport fluid. Reduced transport fluid was manufactured as below in Table 3. Using a spectrophotometer set at λ= 550 nm, absorbance of the suspension was read and adjusted to be between 0.2 - 0.4 to equate to 10^8 cfu/mL.

Table 3 Formulation for the manufacture of reduced transport fluid. Composition per litre of deionised water, pH 8.0 ± 0.2 at 25°C

Stock mineral salt solution No. 1	75 mL
Stock mineral salt solution No. 2	75 mL
Ethylenediamine tetraacetic acid (1M solution) (Sigma)	10 mL
Sodium carbonate (8% solution) (VWR)	5 mL
Dithiothreitol (1% solution) (VWR)	20 mL
Resazurin (0.1% solution) (Sigma)	1 mL
Stock Mineral Salt Solution No 1 Composition per 100 mL of deionised water	
Dipotassium hydrogen phosphate (VWR)	0.6 g
Stock Mineral Salt Solution No 2 Composition per 100 mL of deionised water	
Sodium chloride (VWR)	1.2 g
Ammonium sulphate (VWR)	1.2 g
Dipotassium hydrogen phosphate (VWR)	0.6 g
Magnesium sulphate heptahydrate (VWR)	0.25 g

Two 500 mL duran flasks containing 250 mL of TSB fitted with screw caps were prepared and autoclaved at 121°C for 15 minutes. When cooled the flasks were inoculated with 3 mL of the 10^8 suspension and incubated depending on organism used for 6 hours at 37°C either aerobically (*S. sanguinis*) with shaking or anaerobically (*V. dispar*) without shaking. To each of the HA coated wells and HA disc containing wells was added 20 μL of the freshly prepared bacterial

culture using separate plates for each of the different species. Using the prepared plates, 180 μL of TSB was added to each of the wells. Plates were incubated dependent on the species being investigated. *S. sanguinis* was incubated at 37°C overnight aerobically with shaking. *V. dispar* was incubated at 37°C overnight anaerobically without shaking.

3.2.2.3 Test Method for the Disruption of the Biofilms

3.2.2.3.1 Disruption Procedure – Hydroxyapatite Discs

Ringers and peptone diluent containing either 0.0% v/v, 0.1% v/v, 1.0% v/v or 5.0% v/v Tween20 (Sigma) was manufactured and dispensed in 10 mL aliquots in McCartney bottles (VWR) and autoclaved at 121°C for 15 minutes. Using sterile forceps a HA disc with biofilm was removed and placed in McCartney bottle. Each disc being placed individually in the bottles (n=8, for each test condition). Hydroxyapatite discs were then exposed to a number of experimental conditions to determine optimum bacterial recovery. Discs were either vortexed at the highest speed for 30 seconds, 1 and 2 mins in each of the test diluents either with or without glass beads to aid in disruption. Each of the columns on the 96-well plate related to different test parameters as below in Table 4. After vortexting, 1 mL was removed and serially diluted to 10^{-6} in R+P. *S. sanguinis* was plated out on tryptone soya agar (TSA) (Oxoid) and incubated at 37°C aerobically for 24-80 hours. *V. dispar* was plated on BHI agar (Oxoid) and incubated at 37°C anaerobically for 48-120 hours.

Table 4 Test conditions for bacterial recovery from HA disc.

Column	Biofilm disruption conditions
1	R+P, vortexed 30 seconds
2	R+P, vortexed 1 minute
3	R+P, vortexed 2 minutes
4	R+P with 0.1% v/v Tween20, vortexed 30 seconds
5	R+P with 0.1% v/v Tween20, vortexed 1 minute
6	R+P with 0.1% v/v Tween20, vortexed 2 minutes
7	R+P with 1.0% v/v Tween20, vortexed 30 seconds
8	R+P with 1.0% v/v Tween20, vortexed 1 minute
9	R+P with 1.0% v/v Tween20, vortexed 2 minutes
10	R+P with 5.0% v/v Tween20, vortexed 30 seconds
11	R+P with 5.0% v/v Tween20, vortexed 1 minute
12	R+P with 5.0% v/v Tween20, vortexed 2 minutes

3.2.2.3.2 Biofilm Disruption – Hydroxyapatite Coated Microtitre Well

Using the same media as the hydroxyapatite disc biofilm disruption method, the biofilms grown on the hydroxyapatite coated wells were removed by disruption with a cocktail stick sterilised at 121°C for 15 minutes. This biofilm disruption device was used to scrape both the base and sides of the wells for differing lengths of time, using a fresh cocktail stick for each well. The disruption device was placed in 10 mL of diluent with 100 μL of broth from the test well. All test

samples were vortexed for 30 seconds and serially diluted and tested as above. Each of the columns on the 96-well plate related to different test parameters as below in Table 5:

Table 5 Test conditions for bacterial recovery from hydroxyapatite coated well

Column	**Biofilm disruption conditions**
1	R+P, scraped 20 seconds, vortexed 30 seconds
2	R+P, scraped 40 seconds, vortexed 30 seconds
3	R+P, scraped 60 seconds, vortexed 30 seconds
4	R+P with 0.1% v/v Tween 20 , scraped 20s, vortexed 30s
5	R+P with 0.1% v/v Tween 20, scraped 40s, vortexed 30s
6	R+P with 0.1% v/v Tween 20, scraped 60s, vortexed 30s
7	R+P with 1.0% v/v Tween 20, scraped 20s, vortexed 30s
8	R+P with 1.0% v/v Tween 20, scraped 40s, vortexed 30s
9	R+P with 1.0% v/v Tween 20, scraped 60s, vortexed 30s
10	R+P with 5.0% v/v Tween 20, scraped 20s, vortexed 30s
11	R+P, with 5.0% v/v Tween 20 scraped 40s, vortexed 30s
12	R+P with 5.0% v/v Tween 20, scraped 60s, vortexed 30s

3.2.2.4 Saliva Inocula and Bioplate Recovery Correlation

To correlate the predominant oral genera found in donated saliva to that found in the Bioplate after incubation, traditional microbiological culturing techniques were employed. Using the method stated previously in chapter 2, HA coated microtitre plates were generated. Bacterial enumeration was determined on,

Veillonella agar (VA- composition per L: 5 g Tryptone (Sigma-Aldrich), 3 g yeast extract (Oxoid), 0.75 mL sodium thioglycollate (Sigma-Aldrich), 0.002 g basic fuchin (Sigma-Aldrich), 2.4 mL 60% sodium lactate (Fisher), 15 g Agar (Oxoid)), Mitis Salivarius agar (MSA- Europa Bioproducts), Blood agar + 7% defibrinated horse blood (BA – Oxoid), *Fusobacterium* agar (FA- Anaerobe Systems, CA, USA), and Fastidious anaerobic agar (FAA- Oxoid) and FAA supplemented with 0.0005% w/v metronidazole (FAAM- composition per L: 45.6 g FAA (Bioconnections), 50 mL defibrinated horse blood (Southern Group Laboratory), 1 mL 2.9 mM metronidazole (Sigma-Aldrich). BA and MSA incubated aerobically at 37°C for 4 days; the remaining media was incubated at 37°C for 7 days.

Two 500 mL flasks containing 250 mL of TSB were prepared, to which 3 mL of donated saliva was added. One bottle was incubated aerobically with shaking (200 rpm) for 6 hours at 37°C. The second bottle was incubated anaerobically without shaking at 37°C. One millilitre of the donated pooled saliva was serially diluted to 10^{-8} cfu/mL in R+P. One hundred microlitres of each dilution was spread plated in duplicate on each test medium. BA and MSA incubated aerobically at 37°C for 4 days; the remaining media was incubated at 37°C for 7 days.

After incubation of the pooled saliva and media, 1 mL was removed from each of the flasks and serially diluted and spread plated on the test media, as stated above. After 6 hours incubation, the 2 x 250 mL of inoculated TSB, one incubated

aerobically the other anaerobically were combined and gently mixed. Twenty microlitre aliquots of the bacterial suspension were added to each of the HA-coated test wells. Plates were incubated for 24 hours aerobically with shaking (200 rpm). Subsequently, using the biofilm disruption method stated previously, a sample of the bacteria present on the HA surface was serially diluted and spread plated as above.

3.2.2.5 Molecular Analysis

From the work carried out in section 3.2.2.4. using the two 250 mL TSB that were inoculated with pooled saliva, with one flask being incubated at 37°C aerobically with shaking and the other flask incubated at 37°C anaerobically without shaking. One millilitre was removed from each of the flasks and serially diluted and spread plated on BA. Colonies with differing morphologies were selected for 16S rDNA sequence analysis. From the 10^{-1} plated dilution, 8 colonies were selected using a sterile applicator stick and suspended in 100µL of mastermix (78.7 µL dH_2O, 10 µL 10x NH_4 buffer (Bioline), 5 µL 50 mM $MgCl_2$ (Bioline), 2 µL10 mM dNTPs (Promega Life Sciences), 2 µL of 27f and 1492r primers (10 mM, Sigma-Aldrich), 0.3 µL BioTaq (Bioline)).

Subsequently, the mix was run on the following thermocycling programme to amplify 16S rDNA. Samples were held at 94°C for 5 minutes followed by 29 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 5 minutes. PCR products were confirmed on a 1% w/w agarose gel where the product was

expected to be around 1.5 kbp. Therefore, a 100 bp ladder (Promega) as a size marker was used. Product was cleaned using PCR purification kit (QIAGEN).

To sequence the PCR product, a PCR reaction mix was manufactured which contained, 1 µL dH_2O, 3 µL of amplified PCR product, 1 µL 357f primer (5 pmol/µL), 2 µL BigDye DNA Sequencing kit (first diluted 1 in 4 with 5x SEQ buffer (400 mM TRIS and 10 mM $MgCl_2$ – Applied Biosciences). Subsequently the mix was run on the following thermocycling programme which contained 99 cycles of being held at 95°C for 10 seconds, 50°C for 5 seconds and then at 60°C for 4 minutes. DNA was then subjected to ethanolic precipitation and resuspended in 20 µL of TSR (Template Suppresser Reaction – Applied Biosciences) and then used for sequencing. The sequences generated were checked using the CHROMAS program and entered into BLAST (http://www.ncbi.nlm.nih.gov/Blast/) and RDP (www.cme.msu.edu/RDP/html/index.html) for sequence matches.

3.2.3 Effect of Media on Biofilm Viability

Due to the results received from the study carried out in section 3.2.2.4, the effect of media on the developing biofilm viability was investigated.

Hydroxyapatite coated microtitre plates were manufactured as reported previously. Donated saliva was collected and incubated in either BHI broth or TSB for 5 hours at 37°C. Subsequently, 20 µL was added to two HA coated microtitre plates. Test medium was prepared in advance and included, artificial

saliva (composition per L, porcine stomach mucins 2.5 g (Sigma-Aldrich), NaCl 3.5 g (Sigma-Aldrich), KCl 0.2g (Sigma-Aldrich), $CaCl_2.2H_2O$ 0.2g (Sigma-Aldrich), Yeast extract 2.0g (Oxoid), Lab lemco powder 1g (Oxoid), proteose peptone 5.0g (Oxoid), 40% urea solution 1.25 mL (Oxoid)), TSB and BHI all manufactured in single, ½, ¼ and $1/10^{th}$ strength. To each well, 180µL of test medium was added (one column = one test media, n=8) and incubated aerobically for 24 hours at 37°C, one plate with shaking, the other without. Subsequently, the plates were washed using the method stated previously and left empty. To each well 200 µL of 10% v/v AlamarBlue™ in TSB was added and re-incubated for a further 30 minutes with shaking (200 rpm), at 37°C.

3.2.4 Bioplate Testing CHX Dose Response

HA coated microtitre plates were prepared using the method stated previously in chapter 2, incorporating the knowledge from this chapter. The wells were inoculated with a mixed salivary culture in BHI, salivary organisms were derived from pooled saliva from 3 donors. Biofilms formed following overnight incubation without shaking at 37°C aerobically. Biofilms were washed twice with BHI using a Nunc-Immunowash 12. Doubling dilutions of 20% v/v CHX were manufactured in dH_2O, test concentrations of CHX ranged from 0.002-2% v/v with dH_2O used as negative control. CHX was applied to the wells for 2 minutes (n=8) and biofilms were washed twice to remove unattached residual CHX. Two hundred microlitres of BHI was added to each well for 4 hours at 37°C without shaking to allow recovery of the organisms. Subsequently, the wells were washed again with fresh BHI containing 10% v/v AlamarBlue™. Following 30 minutes

incubation with shaking (200 rpm) at 37°C, cell viability was determined by measuring fluorescence using a 530 nm excitation filter and 590 nm emission filter. Viability results are recorded as Mean Fluorescence Units (MFU).

3.3 Results

3.3.1 Investigation of HA Surface for Biofilm Development

3.3.1.1 Hydroxyapatite Disc Surface

Using SEM to analyse surface features, which may appear after incubation for 24 hours in one of the test broths, in Figure 21, a rehydrated blank hydroxyapatite disc shows typical features to compare against any surface modifications with the test broths.

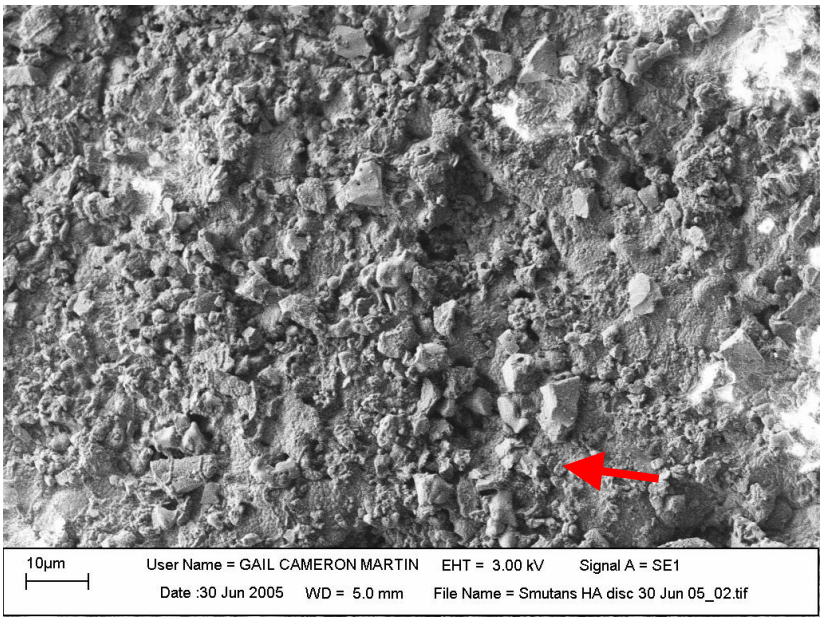


Figure 21 Micrograph of a Hydroxyapatite disc (Bar = 10µm). Image shows rough surface area for bacterial attachment and biofilm development. Red arrow highlights HA material associated with the surface.

Figure 21 shows a number of surface features on the HA disc, including large hydroxyapatite particles on the surface (Figure 21; red arrow). At a higher magnification it was found the surface of the disc contained a “scale-like” surface, (Figure 22; orange arrow). It is believe that this surface is a result of the manufacturing process which compresses hydroxyapatite crystals to form the disc.

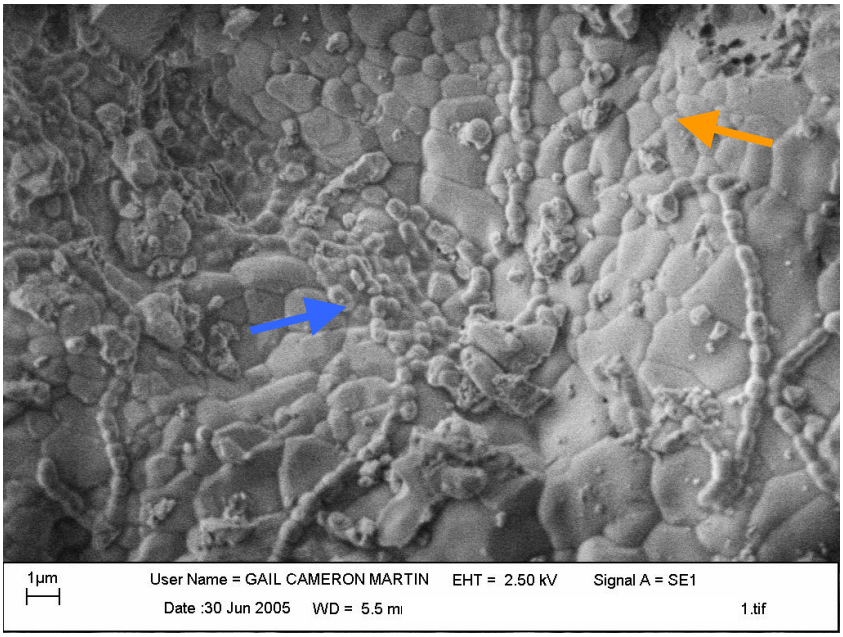


Figure 22 Micrograph of HA disc surface (Bar = 1µm). The blue arrow highlights *Streptococcus mutans* chains associated with the surface. The orange arrow highlights compressed HA crystals. The HA disc is manufactured via compression, and these structures would be expected to be seen.

3.3.1.2 Hydroxyapatite Coated Surface

The surface of the hydroxyapatite coated well was imaged using SEM to understand the surface topography. The surface contains large hydroxyapatite crystals and amorphous HA seen as large globular material as seen in Figure 23.

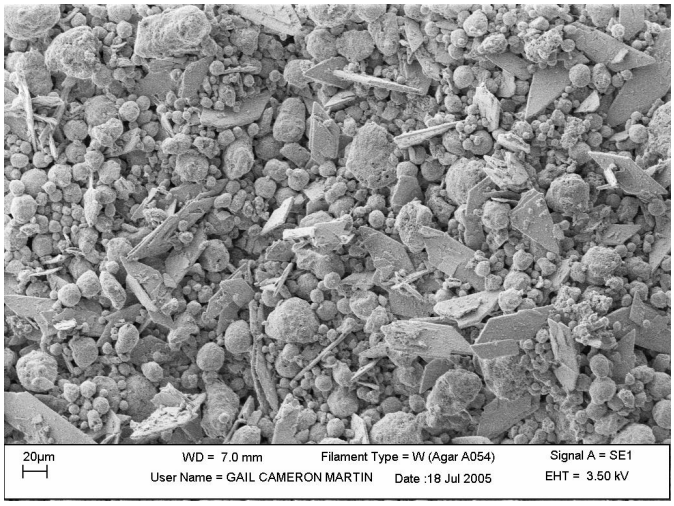


Figure 23 Micrograph of a hydroxyapatite coated well (Bar= 20µm). Image shows two main HA structures, one crystalline the other amorphous. It is hypothesised the amorphous material is created via the manufacture of HA coating on the microtitre plate, from the evaporation of the acetone.

Interestingly, when the HA crystals are dissolved from the surface with 10% w/w citric acid, it was clearly seen the close relationship the HA crystals and amorphous material has with the microtitre surface (Figure 24).

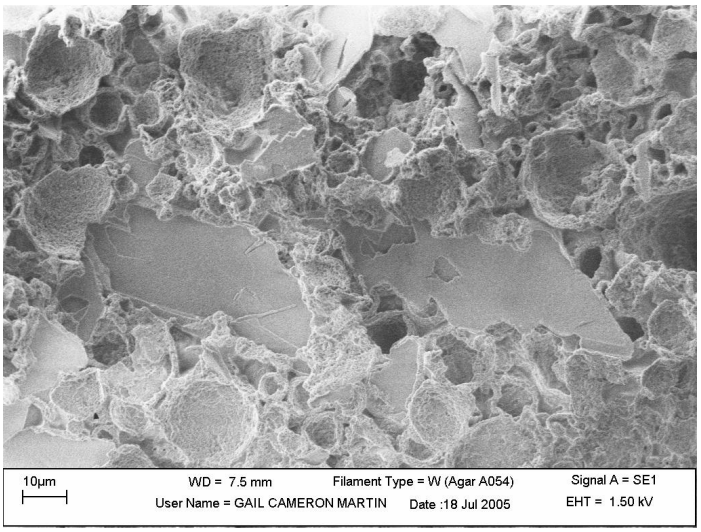


Figure 24 Micrograph showing microtitre plate polystyrene surface after dissolving attached HA. (Bar= 10µm). Image shows where the plastic has melted from the addition of acetone. The structures seen are the imprints left by the HA which are attached to the surface. In the image centre it is clearly seen the outline of two HA crystals.

From Figure 25, it is clearly seen a bacterial lawn growing on the HA crystals.

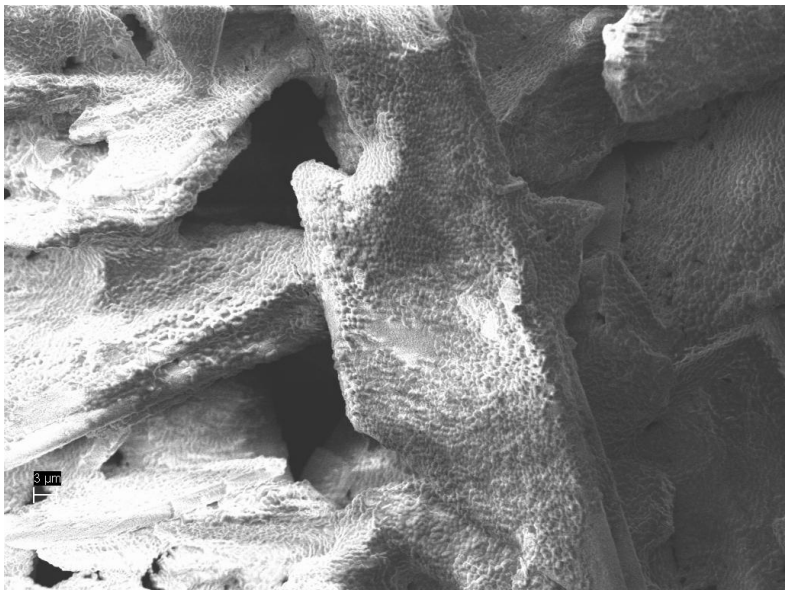


Figure 25. Micrograph of bacterial attachment after overnight incubation with *Streptococcus mutans.* (Bar= 3µm). The organisms have grown around the HA crystals forming a dense bacterial covering.

AFM was also used to determine whether, differences between planktonic grown *S. mutans* which had been dehydrated on to a surface (Figure 26) and those which were grown associated with a surface could be detected (Figure 27). This is important, as using a system such as this, where there is little or no shear, it is necessary to consider whether the organisms are actively attaching to the surface or being deposited.

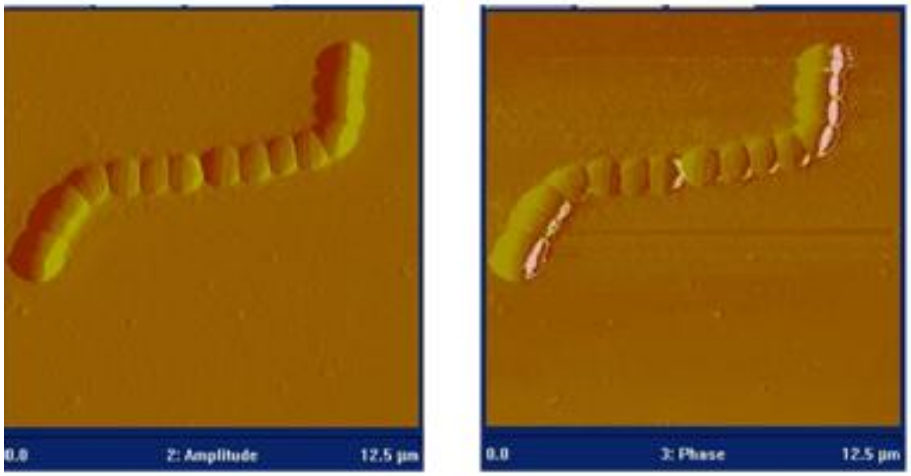


Figure 26. Planktonic *S. mutans* dehydrated onto a glass slide. Utilising amplitude and phase modes show that although the organisms are deposited on the surface by the absence of excreted material 'anchoring' the cells to the surface.

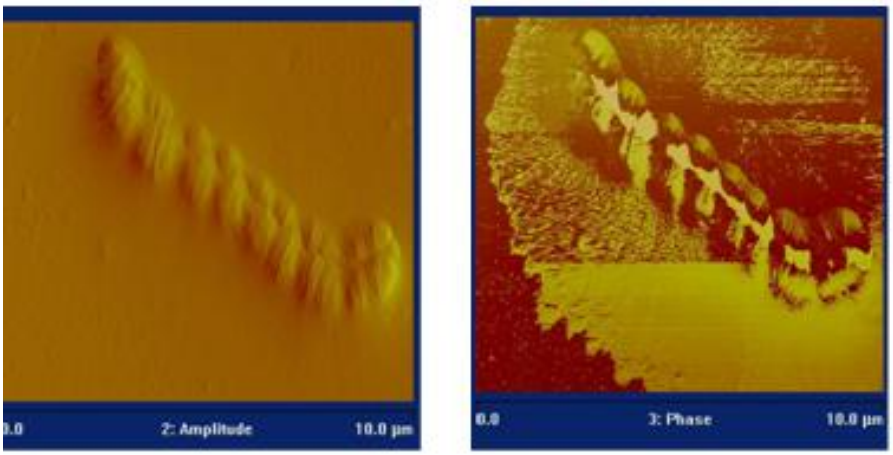


Figure 27 *S. mutans* grown on the surface of a glass slide. Amplitude, reveals the presence of the organisms on the surface, phase mode reveals a material of different 'stickiness' exuding from the organisms attached to the surface.

An excreted material can clearly be seen in Figure 27 (Phase image); this substance is in close relationship to the growing cells. This material was not found in either of the images in Figure 26. It is believed that this material is excreted EPS from the bacterial cells. AFM force studies would have helped

investigate wither these cells were indeed attached to the surfaces, however, due to limitations in the equipment; this aspect of the study was not progressed at this time. The AFM was unable to determine differences in the attachment to the test surfaces due to the rough surface topography. The test surfaces were replaced with glass discs and attachment to this surface was investigated. Using a smoother surface resulted in differences in attachment by planktonic and attached cells to the surface being easily seen.

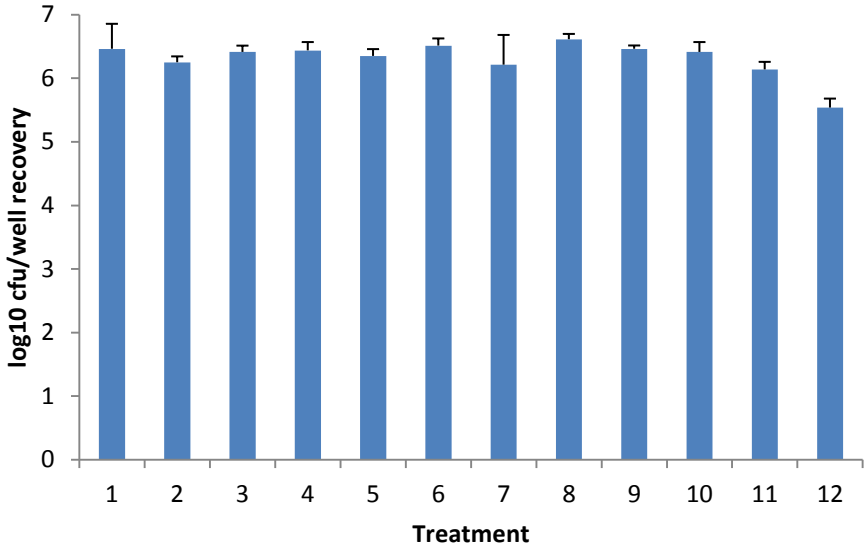
3.3.2 Biofilm Disruption Methodology

Reliability in the recovery of organisms from the bioplate, required an exploratory study to be carried to ensure consistency of bacterial recovery and ensure that the method employed to recovered the organisms revealed the true numbers of organisms available. In this analysis, Tween 20 was employed as an adjunct to the buffer solution to aid biofilm distruption and to allow bacterial separation, for ease of counting. As discussed by Xaio and Stewart (2000), Tween 20 may disrupt the biofilm matrix by interfering with the hydrophobic interactions involved in crossing linking of the structure. Indeed, Walker and Sedlacek (2007) showed that 0.5% Tween 20 was appropriate to reduce bacterial clumping without affecting viability.

3.3.2.1 *Streptococcus sanguinis* Biofilm Disruption Studies

Disruption and recovery from *S. sanguinis* biofilms grown on hydroxyapatite coated wells (n=8) show little variability in recovery, as seen in Figure 28. This

initial study was carried out only once. Recovery using each of the diluents and techniques were similar with an average of approximately $1.5x10^6$ cfu/mL. The only exception was the 5.0%v/v Tween20 when the surface was also scraped for 60 seconds. There was found to be no benefit in scraping the surface for greater than 20 seconds. The inclusion of Tween20 into the medium to aid disaggregation also conferred no benefit. Therefore, all future experiments used the protocol of scraping the well surface for 20 seconds and vortexing in R+P for 30 seconds to remove adhered cells in the absence of Tween20.



Treatment	Media	Time well scraped
1	R+P only	20 seconds
2	R+P only	40 seconds
3	R+P only	60 seconds
4	R+P with 0.1% Tween 20	20 seconds
5	R+P with 0.1% Tween 20	40 seconds
6	R+P with 0.1% Tween 20	60 seconds
7	R+P with 1% Tween 20	20 seconds
8	R+P with 1% Tween 20	40 seconds
9	R+P with 1% Tween 20	60 seconds
10	R+P with 5% Tween 20	20 seconds
11	R+P with 5% Tween 20	40 seconds
12	R+P with 5% Tween 20	60 seconds

Figure 28 *Streptococcus sanguinis* recovery from hydroxyapatite coated well, treatment regimes in table above. Using a number of different levels of Tween20 to aid in biofilm disruption and varying the time wells scraped, it was found that the use of 5.0%v/v Tween20 in the diluent had a negative effect on organism recovery. All other results are comparable with one another. For all experiments n=8.

S. sanguinis $\log_{10}$ recovery from HA coated wells were analysed using a one–way analysis of variance with a factor for treatment, where $p<0.05$.

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	6.85583	11	0.623258	14.97	0.0000
Within groups	3.4975	84	0.0416369		
Total (Corr.)	10.3533	95			

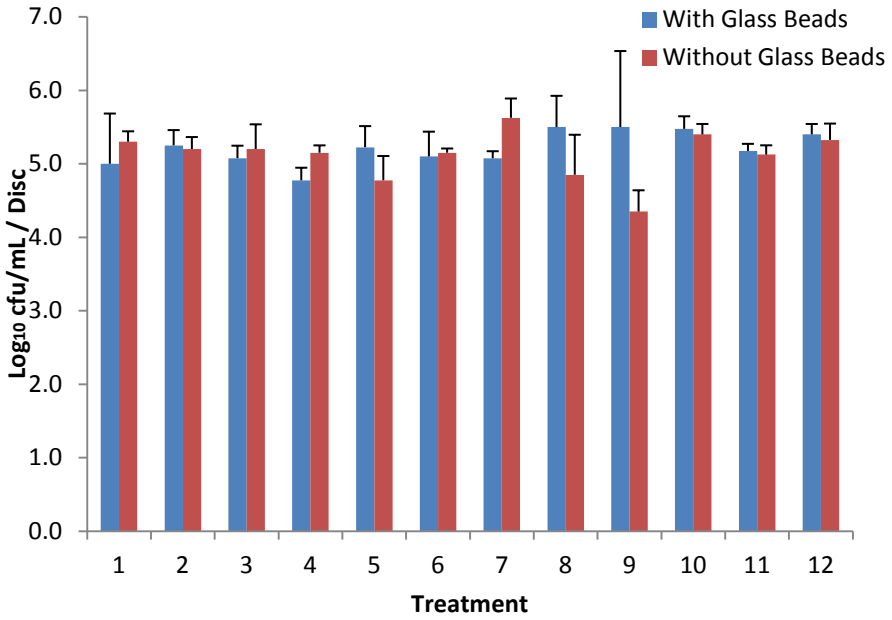
Treatments with the same letter are not significant

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD

Treatment	Count	Mean	Homogeneous Groups
12	8	5.5375	A
11	8	6.1375	B
7	8	6.2125	BC
2	8	6.25	BCD
5	8	6.35	CDE
10	8	6.4125	CDEF
3	8	6.4125	CDEF
4	8	6.4375	DEF
9	8	6.4625	EF
1	8	6.4625	EF
6	8	6.5125	EF
8	8	6.6125	F

Treatment 12 (R+P with 5% Tween 20), was the only treatment to be statistically distinct from the other treatments, however, this was due to the treatment regime negatively impacting bacterial viability. From the initial work carried out above, testing carried out on the hydroxyapatite discs was reduced to n=4 as a reflection on the tight standard deviations across the test plate. Results for recovery on a hydroxyapatite disc can be found in Figure 29.



Treatment	Biofilm disruption conditions
1	R+P, vortexed 30 seconds
2	R+P, vortexed 1 minute
3	R+P, vortexed 2 minutes
4	R+P with 0.1%v/v Tween20, vortexed 30 seconds
5	R+P with 0.1%v/v Tween20, vortexed 1 minute
6	R+P with 0.1%v/v Tween20, vortexed 2 minutes
7	R+P with 1.0%v/v Tween20, vortexed 30 seconds
8	R+P with 1.0%v/v Tween20, vortexed 1 minute
9	R+P with 1.0%v/v Tween20, vortexed 2 minutes
10	R+P with 5.0%v/v Tween20, vortexed 30 seconds
11	R+P with 5.0%v/v Tween20, vortexed 1 minute
12	R+P with 5.0%v/v Tween20, vortexed 2 minutes

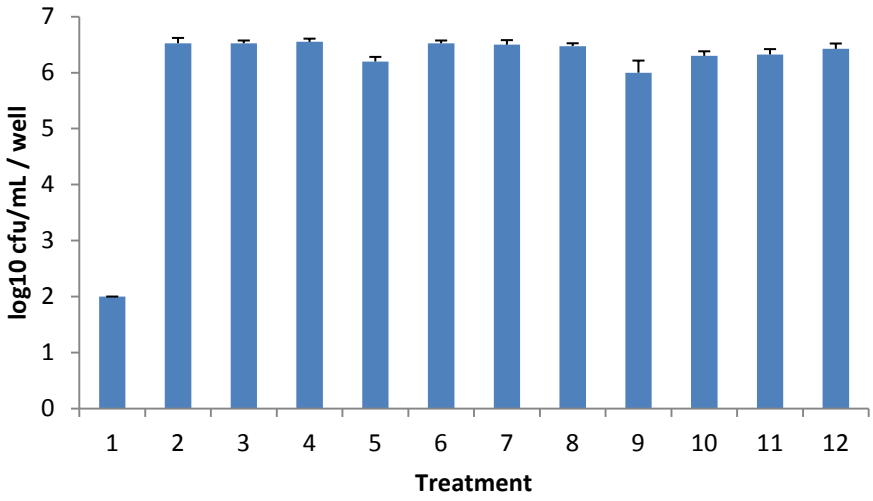
Figure 29 *Streptococcus sanguinis* recovery on hydroxyapatite disc. Test conditions in each of the columns can be found below; figure shows little difference on *Streptococcus sanguinis* recovery using glass beads to disrupt the surface. At the higher levels of Tween20, there was not found a reduction in cell viability.

The results across all the wells show no pronounced benefit to using glass beads to dislodge the biofilms from the hydroxyapatite disc surface; increasing vortexing time also seems to offer little benefit, as was the inclusion of Tween20. With this investigation a reduction in cell viability was not found using the highest concentration of Tween20. There was found a 1 Log_{10} reduction in

recovery using hydroxyapatite discs in comparison to using the hydroxyapatite coated well. In this study however, there was no evidence of a statistical difference between treatments (p=0.3006).

3.3.2.2 *Veillonella dispar* Biofilm Disruption Studies

All results from the *Veillonella dispar* studies (n=4) and the highest concentration of Tween20 were not included in these studies due to the negative results found with the *S. sanguinis* HA-coated microtitre biofilm disruption studies.



Treatment	Biofilm disruption conditions
1	R+P, scraped 0 seconds, vortexed 30 seconds
2	R+P, scraped 30 seconds, vortexed 30 seconds
3	R+P, scraped 60 seconds, vortexed 30 seconds
4	R+P, scraped 120 seconds, vortexed 30 seconds
5	R+P with 0.1%v/v Tween 20, scraped 0 seconds, vortexed 30 seconds
6	R+P with 0.1%v/v Tween 20, scraped 30 seconds, vortexed 30 seconds
7	R+P with 0.1%v/v Tween 20, scraped 60 seconds, vortexed 30 seconds
8	R+P with 0.1%v/v Tween 20, scraped 120 seconds, vortexed 30 seconds
9	R+P with 1.0%v/v Tween 20, scraped 0 seconds, vortexed 30 seconds
10	R+P with 1.0%v/v Tween 20, scraped 30 seconds, vortexed 30 seconds
11	R+P with 1.0%v/v Tween 20 scraped 60 seconds, vortexed 30 seconds
12	R+P with 1.0%v/v Tween 20, scraped 120 seconds, vortexed 30 seconds

Figure 30 *Veillonella dispar* recovery from hydroxyapatite coated wells (n=4). Using a number of different concentrations of Tween20 to aid in biofilm disruption and varying the time wells scraped, it was found that the use of R+P diluent with no Tween20 gave lower recover than that of all other test conditions. Results where no scraping was carried out show comparable recovery to that of the well in which scraping took place.

V. dispar log10 recovery from HA coated wells were analysed using a one-way analysis of varience with a factor for treatment, where $p<0.05$

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	72.0442	11	6.54947	748.51	0.0000
Within groups	0.315	36	0.00875		
Total (Corr.)	72.3592	47			

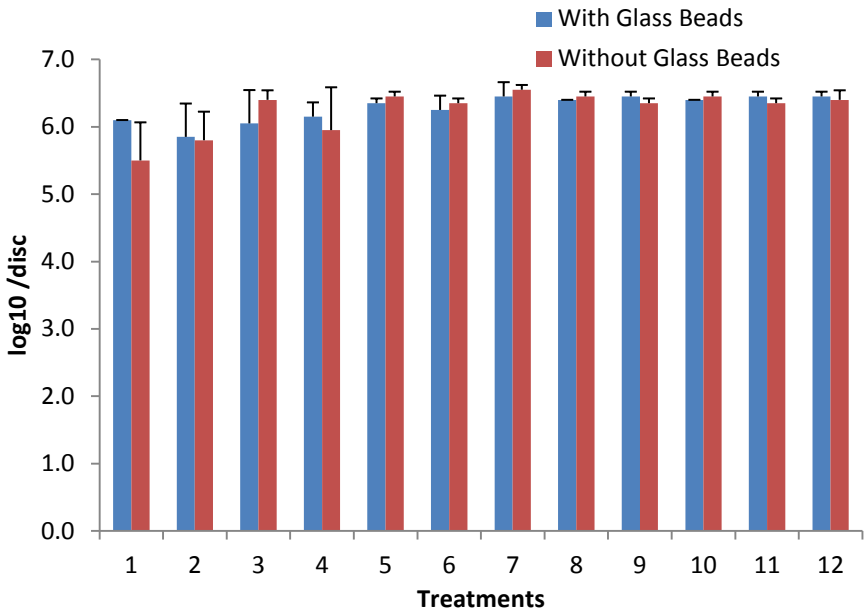
Treatments with the same letter are not significant.

Method: 95.0 percent LSD

Treatment	Count	Mean	Homogeneous Groups
1	4	2.0	A
9	4	6.0	B
5	4	6.2	C
10	4	6.3	CD
11	4	6.325	CD
12	4	6.425	DE
8	4	6.475	E
7	4	6.5	E
6	4	6.525	E
3	4	6.525	E
2	4	6.525	E
4	4	6.55	E

Statistical analysis show that treatment regimes 1 (R+P, scraped 0 seconds, vortexed 30 seconds) and 9 (R+P with 1.0%v/v Tween 20, scraped 0 seconds, vortexed 30 seconds) are statistically distinct. This is due to both these wells not being scraped to aid bacterial removal. Interesting the third treatment leg which was also not scraped (treatment 5) showed directionality to split from treatments, but was statistically similar to treatments 10 and 11.

Veillonella dispar recovery from hydroxyapatite disc again showed that the use of glass beads to help dislodge bacteria from the surface does not increase organisms recovered from the diluent.



Treatments	Biofilm disruption conditions
1	R+P, vortexed 0 seconds
2	R+P, vortexed 30 minute
3	R+P, vortexed 1 minutes
4	R+P, vortexed 2 minutes
5	R+P with 0.1%v/v Tween20, vortexed 0 minute
6	R+P with 0.1%v/v Tween20, vortexed 30 seconds
7	R+P with 0.1%v/v Tween20, vortexed 1 minute
8	R+P with 0.1%v/v Tween20, vortexed 2 minute
9	R+P with 1.0%v/v Tween20, vortexed 0 seconds
10	R+P with 1.0%v/v Tween20, vortexed 30 seconds
11	R+P with 1.0%v/v Tween20, vortexed 1 minute
12	R+P with 1.0%v/v Tween20, vortexed 2 minutes

Figure 31*Veillonella dispar* recovery from hydroxyapatite disc (n=2). Test conditions in each of the columns can be found above; figure shows little difference on *Veillonella dispar* recovery using glass beads to disrupt the surface.

The results from both studies looking at *V. dispar* recovery from hydroxyapatite disc and coated wells show little difference in bacterial recovery after 30 seconds of scraping or vortexing. Therefore, it is recommended that this step in the method need not exceed 30 seconds. As with all the media types a slight reduction in R+P recovery is found. Although this recovery is within the limits of the test, it is still recommended that the diluent formulations contain R+P with

0.1%v/v Tween20. Unlike the results from *S. sanguinis* there was no statistical difference between treatments (p=0.2555).

3.3.3 Saliva Inocula Results

The exploratory results below show Log_{10} bacterial recovery from each of the incubation steps currently involved in the Bioplate method, post-donation from saliva, aerobic and anaerobic incubation, mixing and biofilm development. Two growth media we investigated on their ability to retain mixed populations in the developed biofilm.

Table 6 Bacterial recovery (Log_{10}) using different incubation media (TSB, BHI) after different incubation conditions (from saliva, aerobic, anaerobic incubation, post-mixing, post-biofilm formation)

Broth	**Sample**	**Media (recovery Log10)**					
		Veillonella	**MS**	**BA**	**Fuso.**	**FAA**	**FAAM**
TSB	**Saliva**	7.8	7.5	8.0	5.6	8.2	8.0
	Aerobic	6.4	9.2	9.2	4.3	9.4	9.2
	Anaerobic	6.1	9.3	9.3	4.9	9.3	9.3
	Mixed	6.1	8.3	8.4	3.1	8.3	8.2
	Biofilm	<1	<1	3.4	<1	<1	<1
BHI	**Saliva**	6.9	6.8	6.9	5.2	7.7	7.3
	Aerobic	5.4	8.5	8.9	3.1	8.8	8.7
	Anaerobic	5.4	9.3	9.2	3.7	9.5	9.3
	Mixed	5.5	8.3	8.4	2.5	8.4	8.5
	Biofilm	<1	<1	<1	<1	<1	<1

MS = Mitis salivarius agar, BA = Blood agar, Fuso. = Fusobacterium agar, FAA = Fastidious anaerobe agar, FAAM = Fastidious anaerobic agar with 0.0005% metronidazole.

There was little change in the viable cells recovered following the aerobic and anaerobic incubation steps. However, the recovery of viable cells after incubation of the biofilm in both TSB and BHI reduced complexity compared with the donated saliva (Table 6). After incubation and biofilm development, there was a

gross loss off bacterial recovery, highlighting that the methodology currently employed did not support, either retaining mixed species or subsequent development into a biofilm.

Molecular identification was carried out on isolates taken from the intial incubation step and recovered on BA; 8 distinct colonies of differing morphologies were selected for 16SrDNA. The sequences generated were entered into BLAST. In this preliminary study only 8 colonies were sequenced and 5 samples came back with a positive result following searches, three sequences returned no results due to the short squences. When these sequences were initially entered in to BLAST in 2005 all were identified as uncultured bacterial clones. These sequences were run again through BLAST in Jan 2012 and 5 matched sequences now in the database. All samples came back as *Streptococcu*s spp. In particular, *Streptococcus infantis* ATCC 700779, *S. oralis* ATCC 35037, *S. mitis* NS51, *S. salivarius* ATCC 7073. All results have greater than 93% similarity.

3.3.4 Effect of Media on Biofilm Viability

Previous analyses (3.3.3) revealed that the current methodology employed lost bacterial viability, therefore this study inestigated which incubation and media would optimise bacteria viability and biofilm development in the bioplate. This study was concerned with determining the effect on the strength of media and the

effect of shaking during incubation on biofilm growth. This study utilised donated saliva incubated in BHI, TSB or artificial saliva.

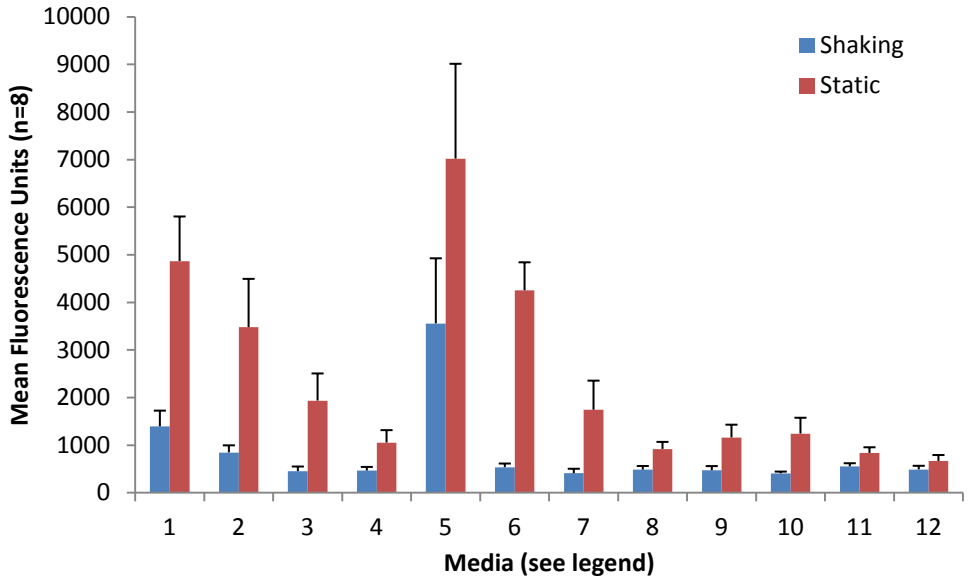


	Figure Legend -Media						
1	TSB – full strength		**5**	BHI-Full strength		**9**	Artificial saliva – full strength
2	TSB – ½ strength		**6**	BHI-½ strength		**10**	Artificial saliva – ½ strength
3	TSB – ¼ strength		**7**	BHI – ¼ strength		**11**	Artificial saliva – ¼ strength
4	TSB – 1/10 strength		**8**	BHI – 1/10 strength		**12**	Artificial saliva – 1/10 strength

Figure 32 reveals that incubating the inoculated hydroxyapatite plate with shaking reduced biofilm viability, this effect was noted across all test media. Artificial saliva at all concentration tested showed the least biofilm viability. TSB gave a good dose response with respect to the concentration tested however BHI half strength medium incubated without shaking gave the best improvement in biofilm viability.

Post-incubation without shaking, was analysed using a one-way analysis of variance with a factor for treatment. There was evidence of difference between treatments ($p<0.05$).

Treatments with the same letter are not the significant.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment   Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
12              8         667.875           A
11              8         835.25            A
8               8         920.0             A
4               8         1054.63           AB
9               8         1161.5            AB
10              8         1242.5            ABC
7               8         1743.88            BC
3               8         1936.38              C
2               8         3477.75               D
6               8         4253.63                E
1               8         4867.88                E
5               8         7020.13                 F
```

Statistical analyses reveal that treatment group 5, gave the best retention of biofilm viability in this study. Therefore going forward the methodology employed will include incubation with full strength BHI broth with the bioplate being incubated statically.

3.3.5 Chlorhexidine Dose Response

Chlorhexidine digluconate dose response indicates that the concentrations required to reduce fluorescence was 0.031% v/v CHX. There was no antimicrobial activity at chlorhexidine levels below 0.016% v/v CHX (Figure 33). Figure 34 shows the visual change from the unreduced blue resazurin to the reduced pink resorfin. Chlorhexidine dose response was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$

```
                          Analysis of Variance
------------------------------------------------------------------------------
Source             Sum of Squares     Df  Mean Square    F-Ratio      P-Value
------------------------------------------------------------------------------
Between groups          6.6011E8     11      6.001E7     760.06       0.0000
Within groups          6.63214E6     84      78954.1
------------------------------------------------------------------------------
Total (Corr.)          6.66742E8     95
```

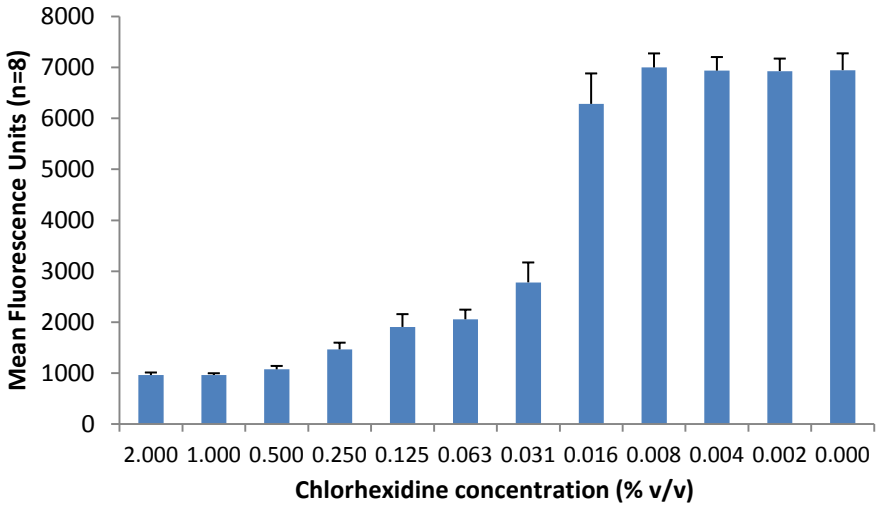


Figure 33. Chlorhexidine dose response against an orally relevant biofilm drived from salivary inoculum and grown in BHI broth incubated statically.

Figure 34. Image of CHX dose response in Bioplate with 10 %v/v AlamarBlue™.

Treatments with the same letter are not significant.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Col_1          Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
1              8         961.25            X
2              8         965.125           X
3              8         1074.13           X
4              8         1465.13            X
5              8         1905.75              X
6              8         2055.88              X
7              8         2781.0                 X
8              8         6282.75                  X
11             8         6926.63                    X
10             8         6935.0                     X
12             8         6944.38                    X
9              8         6997.0                     X
```

Statistical analysis on the bioplate results show good statistical separation of the CHX dose response. It was found that treatment groups 9-11 (0.002-0.008% v/v CHX) did not split from the water control. Treatment groups 1-3 (0.5-2.0% v/v CHX) did not split, where 2.0% v/v CHX was the positive control in this study.

3.4 Discussion

The key attribute of this assay is to demonstrate the antimicrobial efficacy of test agents against orally relevant biofilms, using the initial methodology as discussed in Chapter 2. The bioplate assay has to fulfil many of the required criteria, i.e. orally relevant surface, short contact time, reproducible with high number of replicates against an oral biofilm population. By defining each of the test parameters, a robust and reproducible method for the evaluation of antimicrobials has been developed.

Initial investigations have shown that simple changes to the methodology can improve bacterial coverage of the HA surface. Changing biofilm incubating media from TSB to BHI, from incubating with shaking to incubating without shaking of the inoculated HA plate conferred the greatest improvement of the assay. This was confirmed via SEM imaging of the surface using existing methodology (image not shown) to that of the optimised method. The initial results showed little bacterial recovery after incubation whether in TSB or BHI, giving rise to the hypothesis that incubating the Bioplate without shaking gives the greatest increase in bacterial attachment and biofilm development. In contrast, the protocol for the CBD device states that shaking is required (Ceri *et al.*, 1999). Further work (not shown here) was carried out to investigate any benefit to pelliclising the HA surface with artificial saliva for 30 minutes. This step conferred no benefit to bacterial attachment and development. It is proposed that this is due to the proteins within the BHI broth forming a pellicle over the HA surface, very much like what is seen with salivary protein deposition on HA.

AFM can determine differences between bacteria that are attached and developing into a micro-colony to those which have settled via gravity to the surface. The methodology employed a number of washing steps to remove unattached or loosely bound organisms, therefore, we have shown this technology has the ability to confirm the bacteria attached to the HA crystals are attached.

Two parallel studies were undertaken that investigated the use of a defined inoculum based on the work by Marsh (1985) to that of pooled donated human saliva. Initial molecular analysis suggests the existence of uncultured bacterial clones within the donated saliva, which may add to biofilm complexity, therefore, more closely reflecting *in vivo* biofilms. However, it has been shown previously that the complexity of the microbiota is lost when using *in vitro* systems (Pratten *et al*., 2003) and there are possible problems when using such inocula in maintaining reproducibility. Therefore, to further aid in the refinement of the model a defined inoculum was investigated. It has been identified that the inocula should contain precise quantities of each strain to prevent overgrowth and competition amongst species. It is envisaged that it could be possible to change the composition of the mixed consortia in order to replicate the microbiotas which will be relevant to the three disease states under investigation. Such studies on shifting the microbiota with dual and microcosm oral biofilms have been successfully carried out previously (Dalwai *et al*., 2006).

CHAPTER 4: Standardisation of a Reproducible Biofilm Model

4.1 Introduction

Building on the initial research carried out in chapter 3, optimising the standard Bioplate methodology, the investigations carried out in this chapter look to maximise the model usability, standardisation and stability. The use of microcosm model systems, i.e. using plaque or saliva, has been advocated for many scenarios and has many advantages when trying to replicate the *in vivo* situation. However, issues with reproducibility arise with using a microcosm, as only a limited number can ever be obtained and samples from different geographical locations would differ hugely. Furthermore, as stated previously, it has been studied and reported by Pratten (2003), "…that an inoculum with a diverse bacterial population resulted in biofilms which appeared to contain a far less diverse microbiota, as a result possibly of selection processes inherent in modelling." This in turn, could have an adverse effect on the reproducibility of the developing biofilms within the model system, adversely effecting results. Thus, even with a microcosm inoculum, a reduction in speciation can occur in an *in vitro* system. Therefore, to overcome these difficulties, a defined inoculum was investigated, based on its relevance to oral biofilms and ease in culturability, utilising reduced bacterial species that could be easily monitored. This choice of species for the inoculum was established on the research carried out by McKee (1985) and discussed in chapter 2. The defined inoculum has been utilised in a number of previous studies investigating oral bacteria metabolism (Bradshaw *et al.*, 1994a/b), effect of environmental pH (McDermid *et al.*, 1986; Bradshaw *et al.*, 1989) and effect of antimicrobial agents (McDermid *et al.*, 1987, Bradshaw *et al.*, 1993).

As discussed in chapter 2, the organisms utilised by McKee (1985) were, *Streptococcus mutans* ATCC 2-27351, *Streptococcus sanguis* NCTC 7865, Streptococcus *mitis* EF 186, *Actinomyces viscosus* WVU 627, *Lactobacillus casei* AC 413, *Neisseria sp*. A 1078, *Veillonella alkalescens* ATCC 17745, *Bacteroides intermedius* T 588 and *Fusobacterium nucleatum*, NCTC 10593. Due to the unavailability of some of these organisms, they have been replaced for use in the Bioplate with more easily available representative NCTC strains, *S. mutans* NCTC 10449, *S. sanguinis* NCTC 10904, *S. mitis* NCTC 12261, *A. viscosus* NCTC 10951, *L. casei* var. *rhamnosus* NCTC 10302, *N. mucosa* var. *mucosa* NCTC 10774, *V. dispar* NCTC 11831, *P. melaninogenica* NCTC 11321 and *F. nucleatum* NCTC 10562.

When carrying out *in vitro* biofilm model studies, set-up, organism growth and sample preparation can be as long as, or longer than, the actual analysis carried out on the biofilm. To try and increase the through-put of this model system, the ability to prepare a frozen defined inocula which can be kept until required, would decrease model preparation by 7 days for organism growth. For this protocol this was investigated using the organisms frozen (-80°C) in glycerol, either within prepared Bioplates or within 2 mL tubes waiting to be dispensed onto HA-coated mictotitre plates. Glycerol's cryoprotection properties were first reported over 60 years ago (Polge *et al.,* 1949), since then it has been further studied to identify the optimal concentrations for bacterial cell preservation, these range from 2-55% (Harrison, 1956; Hollander and Nell, 1954; Howard, 1956; Postgate and Hunter, 1961).

In this model system AlamarBlue® is utilised to measure cell viability. AlamarBlue® is based on the compound resazurin, first introduced by Guilbault and Kramer (1964). When placed in a reducing environment AlamarBlue® is reduced to resofurin as shown in Figure 35. Resofurin, when in its reduced form will fluoresce, which can be monitored at 530 nm – 560 nm excitation wavelength and 590 nm emission wavelength (AlamarBlue Product data sheet). It is this change in fluorescence which is used as a viability indicator of both planktonic and biofilm organisms.

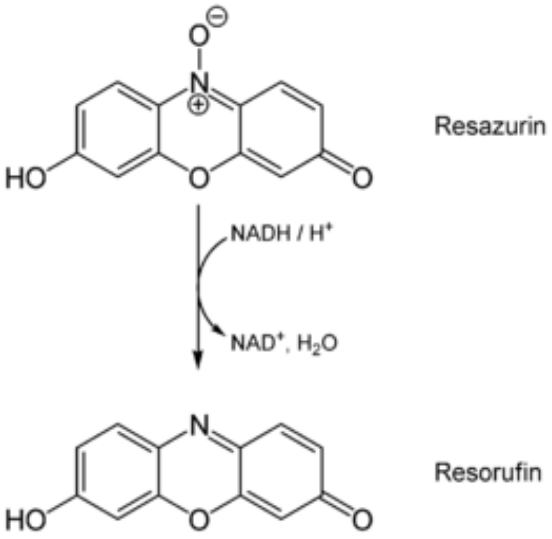


Figure 35 Schematic of resazurin reduction to resorufin

Using the diaphorase-resazurin pathway, Mashige *et al*., (1976) found that the released hydrogen from NADH (as in Figure 35) would reduce resazurin. However, it has also been shown that a number of reductant pathways where NADH is reduced can also affect resofurin generation, such as lactic acid dehydrogenase pathway (Guilbault and Kramer, 1964).

Resazurin has been widely used to assess cell viability post-exposure to antimicrobial agents (Bürgers *et al.,* 2009; Mariscal *et al.,* 2008; Toté *et al*., 2009). To decrease assay time, it has been noted by a number of investigators that having greater than $1x10^5$ CFU present, will help decrease exposure time to resazurin as the metabolic activity will initiate the change in fluorescence faster (O'Brien *et al*., 2000; Shiloh *et al*., 1997; Peters *et al.,* 2008). Previous studies have tried to use various rapid methods to quantify bacterial numbers to the same detection limit currently available with standard culturing techniques. This detection level could be generally stated as $10\text{-}10^2$ CFU. Yamashoji *et al.,* (2004) investigated the possibility that bioluminescence or chemical luminescence could be utilised to quantify total viable counts. Unfortunately the authors found under certain assay conditions a correlation between CFU and luminescence could not be found. Marciscal *et al.* (2009), tried again to correlate total viable count and viability fluorescence, but this time utilised resazurin. In this investigation they found that the resazurin proved to be sufficient for measuring reductions in cell viability of 5-log CFU. However, this too lacked the specificity that is currently found with tradition plating techniques for investigating total viable counts. The authors do discuss that "…..since each microorganisms has different resazurin kinetics it is necessary to generate a standard curve for each assay."

In this chapter, the generation of a standard inoculum to generate standard biofilms will be investigated. The long term storage of this inoculum and the correlation between viable cells within this inocula and fluorescence will be determined.

4.2 Materials and Methods

4.2.1 Species Evaluation

4.2.1.1 Correlation between Absorbance and CFU

Ringers and Peptone (R+P) diluent was manufactured and dispensed into 9 mL and 10 mL aliquots. Blank control readings were taken using R+P diluent in a spectrophotometer set at λ=600 nm and the readings blanked to the R+P control. The same blank was used throughout the experiment.

The organisms used in this study were *S. mutans* NCTC 10449, *S. sanguinis* NCTC 10904, *S. mitis* NCTC 12261, *A. viscosus* NCTC 10951, *L. casei var. rhamnosus* NCTC 10302, *N. mucosa* var. *mucosa* (var. heidelbergensis) NCTC 10774, *V. dispar* NCTC 11831, *P. melaninogenica* NCTC 11321 and *F. nucleatum* NCTC 10562. Streptococcal species were grown on TSA and incubated at 37°C aerobically for 48 hours. The remaining organisms were grown on BA and incubated at 37°C anaerobically for 5-7 days. Representative colonies were removed and placed in 10 mL of R+P; this was repeated until a density equivalent to 2.0 McFarland standard was reached. Diluent was vortexed to ensure sample was completely homogenised. One millilitre sample was removed and placed in a cuvette and placed in the spectrophotometer set at λ=600 nm. Absorbance was measured and recorded. Five millilitres of the initial stock was dispensed and further diluted to an absorbance of 1.0. Once reached, the sample was further diluted in 0.1 Abs increments and plated on appropriate agars for each organism and incubated as appropriate. For absorbance increments from 1.0-0.6,

serial diluting of the sample was carried out to 10^{-10} in R+P, for absorbance increments between 0.5 - 0.1, samples were serially diluted to 10^{-8} in R+P. Post-incubation the relationship between absorbance and cfu/mL was calculated. This calculation was to ensure that all the microorganisms used in the defined inoculum were added at the correct concentrations.

4.2.1.2 Growth Curves

Using the organisms outlined in 4.2.1.1 and incubated as previously discussed in this chapter. Representative colonies were removed and placed in BHI broth to a density equal to 0.5 McFarland standard. Using 9 mL aliquots of BHI serial dilutions were generated to 10^{-9}. One hundred microlitres of each dilution was plated on BA in duplicate and incubated at 37°C either aerobically or anaerobically for between 2-7 days (species dependent).

Using an uncoated 96-well microtitre plate each of the dilutions were added to each column (where n=8). Growth was measured using an automatic microtitre plate reader set for absorbance at 450 nm using BHI as a blank. Readings were taken every 30 minutes for 10-20 hours. The plate reader was set with shaking to reduce settling at 37°C for the duration of the experiment. Each dilution was plated on appropriate agar to give initial counts for each of the absorbencies. Readings were taken at 450 nm due to the limitations of the microtitre plate reader.

4.2.2 Inoculum Stability

4.2.2.1 Generation of Inoculum for Long-Term Storage

Using the data generated in the previous section, each of the concentrations of the organisms were made in R+P. The following bacterial concentrations were generated, following the methods previously discussed in chapter 2, *S. mutans* ($1x10^7$ cfu/mL), *S. sanguinis* ($1x10^8$ cfu/mL), *S. mitis* ($1x10^{10}$ cfu/mL), *A. viscosus* ($1x10^6$ cfu/mL), *L. casei* ($1x10^9$ cfu/mL), *N. mucosa* var. *mucosa* ($1x10^6$ cfu/mL), *V. dispar* ($1x10^9$ cfu/mL), *P. melaninogenica* ($1x10^7$ cfu/mL) and *F. nucleatum* ($1x10^8$ cfu/mL). This was defined using the absorbance graphs in Figure 36. One hundred microlitres of each organism was added to a 2 mL sterile tube, 16 tubes were filled. Each container was vortexed at 8000 rpm for 10 minutes and the supernatant was removed. One millilitre of R+P with 5% glycerol was added to 8 tubes, 1 mL of R+P with 10% glycerol was added to the remaining 8 tubes. Seven of each of the samples were placed in a -80°C freezer. Using the freshly filled tube, this was used to calculate initial cfu/mL bacterial counts.

4.2.2.2 Stability Testing of Manufactured Inoculum

Viable counts were taken at various time points to determine what expiry date should be assigned to the defined inoculum. Viable counts were taken at the following time points whilst being stored at -80°C; initial (as discussed above), and at 1, 2, weeks, then 1, 2, 3, months. At each time point a sample vial was removed from -80°C storage and allowed to come to room temperature. To each vial was added 1 mL of BHI to resuspend the organisms. One millilitre was

removed and added to 9.0 mL R+P. This was vortexed to ensure homogeneity of sample (this will equal a $1x10^{-1}$ dilution). This was subsequently serially diluted to $1x10^{-10}$ and a 100 µL sample plated out in duplicate on appropriate agar and incubated as appropriate.

4.2.3 Bioplate Storage

To allow the development of an 'off-the shelf' biofilm model, understanding the stability of the organisms during storage is paramount to ensure retention of bacterial viability and subsequent biofilm development. The research carried out here, investigated the stability of the organisms for 3 months in glycerol, on a HA coated microtitre plate.

4.2.3.1 Stability of Organisms in a HA coated Microtitre Plate

Hydroxyapatite plates were manufactured as in chapter 2, using the inoculum generated in section 4.2.2.1, 1 mL of each of the organisms were added to 30 mL sterile container, to this was added 9 mL of either 10 or 20% v/v glycerol. An exception was *N. mucosa*, which was manufactured to $1x10^{6}$ cfu/mL to ensure detection above the limit of the agar plating methodology. To each well on the HA coated microtitre plate was added 20 µL of test inoculum containing glycerol, assuming dilution to 5% and 10% v/v respectively. For each time point a separate microtitre plate was utilised to ensure no interference with continual removal from storage, however, the 5% and 10 % v/v glycerol were tested on the same plate. Therefore, 6 HA coated plates are stored. At each time point, 1 week, 2 week, 1

month, 2 months and 3 months a plate is removed and following the methodology for bacterial recovery from HA-coated microtitre plates in chapter 3, the organisms were recovered by adding 180 µL BHI and plated on BA incubated aerobically 37°C and modified BA containing haemin, manufactured as previously, incubated anaerobically at 37°C. This was done due to the very small sample size available in each well, where it would be impractical to test for all species in this first evaluation.

4.2.4 Correlation of Mean Fluorescence Units

4.2.4.1 Planktonic Cells

The organisms used in this study were *S. mutans* NCTC 10449, *S. sanguinis* NCTC 10904, *S. mitis* NCTC 12261, *A. viscosus* NCTC 10951, *L. casei* var *rhamnosus* NCTC 10302, *N. mucosa* var *mucosa* (var. Heidelbergensis) NCTC 10779, *V. dispar* NCTC 10831, *P. melaninogenica* NCTC 11321 and *F. nucleatum* NCTC 10562. Streptococcal species were grown on TSA and incubated at 37°C aerobically for 48 hours. The remaining organisms were grown on BA and incubated at 37°C anaerobically for 5-7 days. Representative colonies were removed and placed in 10 mL of R+P. This was repeated. The density was measured by removing 1 mL sample and placing in a cuvette and placing in a spectrophotometer set at λ=600 nm. Absorbance was measured and recorded to generate a stock solution of $1x10^8$ cfu/mL, using the generated data in Figure 36. One millilitre of each of the stock solutions were added to 200 mL BHI or a modified BHI broth (37g BHI, Merck; 0.5 mL haemin, Sigma (manufactured by

adding 200 mg haemin in 50 mL 1M potassium hydroxide with 50 mL ethanol); 0.2 mL vitamin K, (Sigma; manufactured by adding 0.15g vitamin K with 30 mL ethanol); 1 L deionised water. This media was used for the growth of *Prevotella* sp. All organisms, dependent on requirements were incubated either aerobically with shaking at 37°C or anaerobically without shaking at 37°C.

One millilitre samples were removed from the broths after 2, 4, 6, 24 and 48 hours post-inoculation. The sample was placed in 9 mL R+P and serially diluted in 9 mL R+P to 10^{-8}. One millilitre of each dilution was plated in duplicate on either TSA for streptococcal species and incubated at 37°C aerobically for 48 hours; the remaining organisms were grown on BA and incubated at 37°C anaerobically for 5-7 days. One millilitre sample was also removed at each time point and placed in a cuvette and placed in a spectrophotometer set at λ=600 nm and absorbance recorded.

One hundred microlitre aliquots (in triplicate) were taken and placed in an HA-coated microtitre plates, to which was added 20% v/v AlamarBlue® in BHI. Fluorescence was measured using a microtitre plate reader using a 530 nm excitation filter and a 590 nm emission filter. Results were recorded as mean fluorescent units.

4.2.4.2 Biofilm Cells

Using the data generated in the previous section, each of the concentrations of the organisms were made in R+P. The following organism concentrations were

generated, *S. mutans* ($1x10^7$ CFU/mL), *S. sanguinis* ($1x10^8$ CFU/mL), *S. mitis* ($1x10^{10}$ CFU/mL), *A. viscosus* ($1x10^6$ CFU/mL), *L. casei* ($1x10^9$ CFU/mL), *N. mucosa* var. *mucosa* ($1x10^6$ CFU/mL), *V. dispar* ($1x10^9$ CFU/mL), *P. melaninogenica* ($1x10^7$ CFU/mL) and *F. nucleatum* ($1x10^8$ CFU/mL). This was defined using the absorbance graphs in Figure 36, where n=8.

One millilitre of each organism was added to a sterile container and vortexed to ensure fully mixed. Using a multichannel (x8) pipette, 20 µL of the inoculum was added to a pre-prepared HA coated microtitre plate following the method described in chapter 2. Subsequently, to each well was added 180 µL of BHI, the plate was then placed in a 37°C incubator aerobically for 24 hours, without shaking.

The plate containing the test agents were prepared in advance, CHX (Sigma) was used in this study at 0.2, 0.02 and 0.002% v/v, with deionised water as a negative control. To each column on an uncoated microtitre plate, 200 µL of the test agent was added. In this study, the dose response was repeated on the same plate, where the second dose response would be utilised to investigate cfu/biofilm.

Taking an inoculated Bioplate that had been incubated overnight, exhausted media from each well was removed using a Nunc-Immunowash 12 manual plate washer fed with BHI and attached to an effluent bottle via a vacuum pump. Wells were washed (x2) using the Immunowash to remove unattached or loosely bound bacterial cells, thus leaving the wells of the Bioplate empty. Using a 96-Transtar

pipette (Corning, Sigma-Aldrich) and the uncoated 96-well plate, 200 µL of the test agents were transferred to the now empty Bioplate and incubated at 37°C for 2 minutes while shaking. Subsequently, using the Nunc-Immunowash, each well was washed (x2) with sterile BHI to remove traces of the test agent leaving the Bioplate empty. Using an 8x multichannel pipette, 200 µL of sterile BHI was added to each well on the plate and re-incubated for 4 hours at 37°C. To measure the viability of the biofilm, 10% v/v solution of AlamarBlue™ in BHI was prepared. After the second incubation, the Bioplate was removed from the incubator and each well washed twice with sterile BHI. Using an 8x multichannel pipette, 200 µL of the 10% v/v AlamarBlue™ solution was added to each well. The plate was further incubated for 30 minutes at 37°C with shaking. Fluorescence was measured using a microtitre plate reader using a 530 nm excitation filter and a 590 nm emission filter. Results were recorded as mean fluorescence units (MFU) where n=8.

4.3 Results

4.3.1 Correlation between Absorbance and CFU

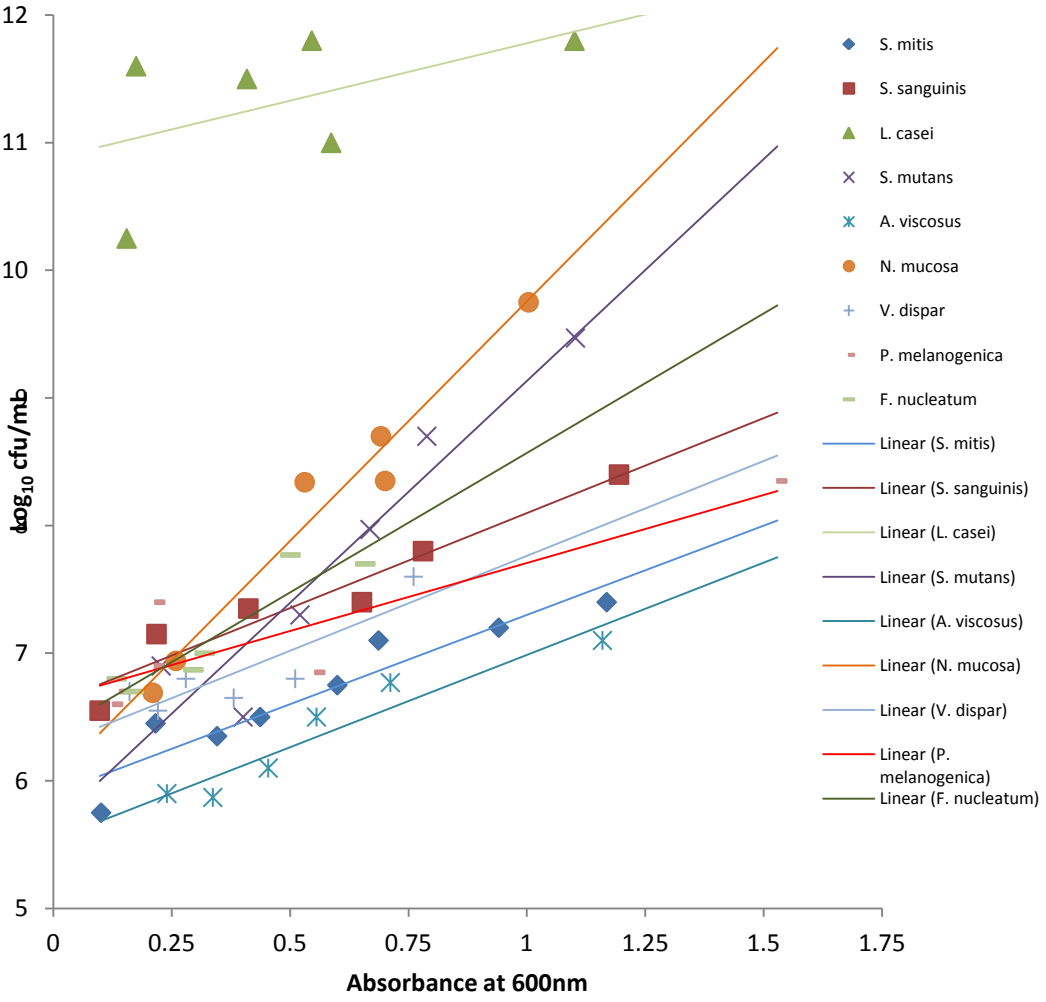


Figure 36 Results showing linear correlation between CFU and absorbance at 600nm.

The results in Figure 36 show the relationship between CFU counts and absorbance taken over one time point. A linear relationship is shown in this study, if this analysis was carried out with respect to time, a linear relationship would not be expected, a typical bacterial growth curve would be anticipated.

4.3.2 Growth Curves

Of all the species investigated, *S. mutans*, *S. mitis*, *A. viscosus*, *L. casei* var. *rhamnosus*, *N. mucosa var. mucosa* (var. heidelbergensis), *P. intermedia* and *F. nucleatum* only the results for the *Streptococcus sanguinis*, *Veillonella dispar* and *S. mitis* will be shown here due to space. The results show that lag phase depends on the initial bacterial concentration of the planktonic cells. It is hypothesised that this is due to the faster utilisation of food source by the organisms contained within the medium.

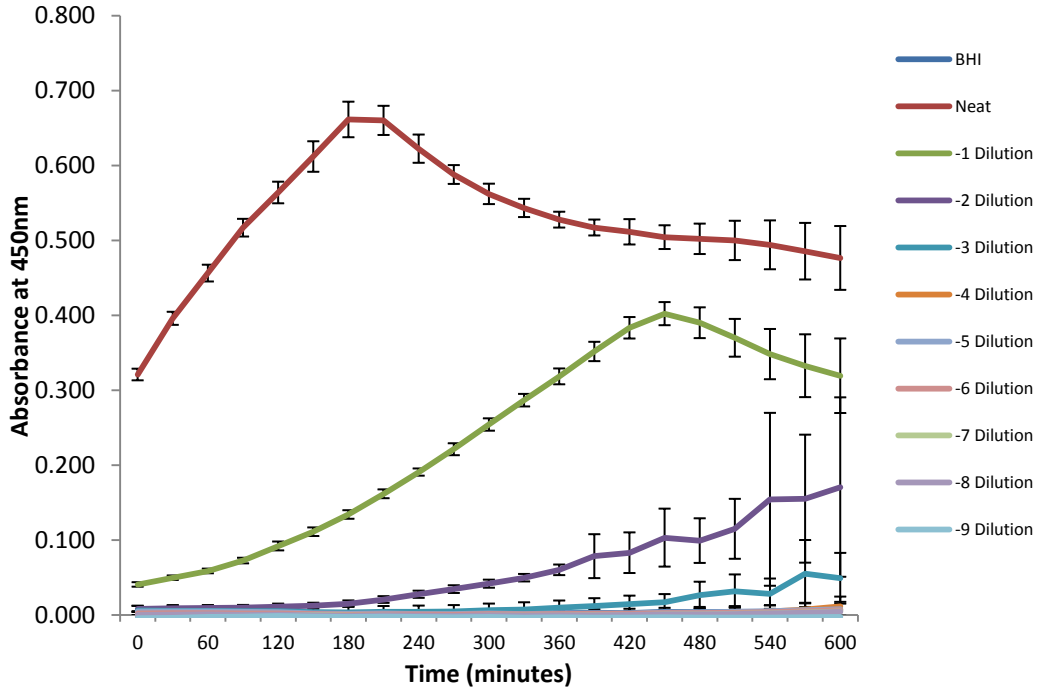


Figure 37 Graph showing *S. sanguinis* differences in growth curves dependent on initial bacterial starting concentration (n=8).

Figure 38 shows *V. dispar* has a shorter exponential phase that *S. sanguinis* as in Figure 37. To ensure a defined population is representative of supragingival plaque these different growth rates will need to be considered.

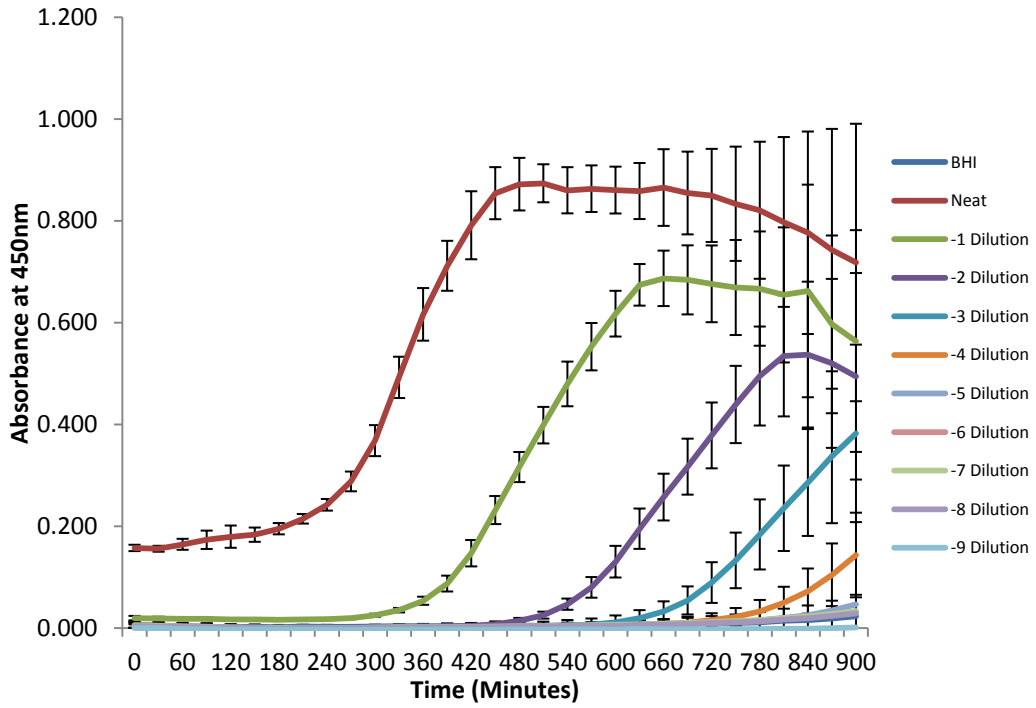


Figure 38 Graph showing *V. dispar* differences in growth curves dependent on initial bacterial starting concentration (n=8).

Growth curves in microtitre plate results were similar to that seen for all species tested, in that the initial starting concentration affected the length of time take to reach stationary and death phase.

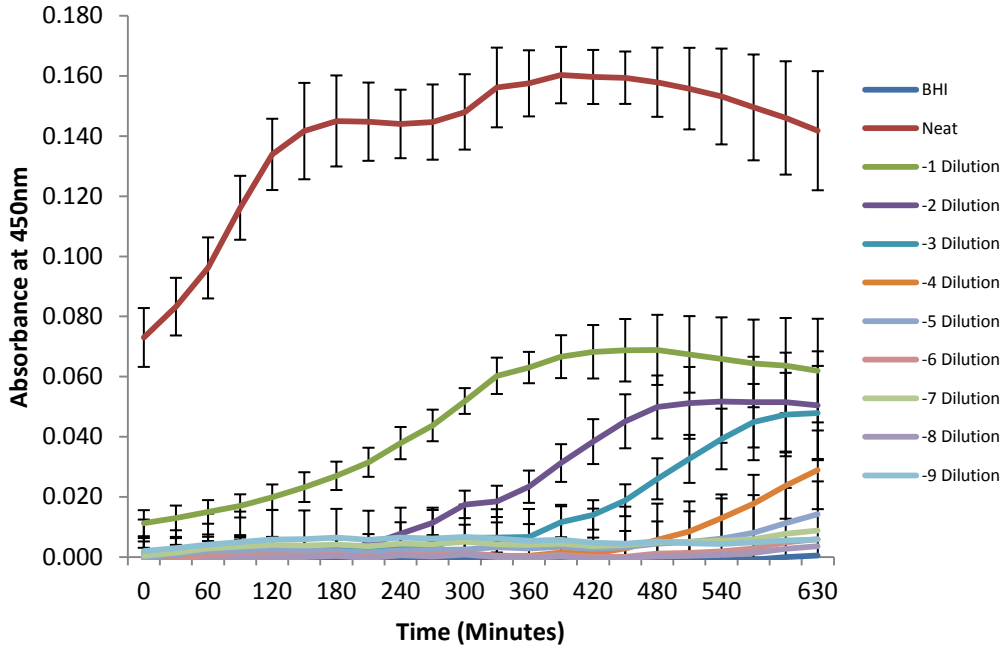


Figure 39 Graph showing *S. mitis* differences in growth curves dependent on initial bacterial starting concentration (n=8).

4.3.3 Stability Testing of Inoculum

The results in Table 7 show the recovery of the organisms in 5% glycerol held at -80°C for up to three months in 2 mL Eppendorf tubes.

Table 7 Stability of individual organisms held in 5% Glycerol for three months at -80°C, reported in Log_{10}

Organisms (starting concentration)	Initial (post-dilution)	1 week	2 weeks	1 month	2 months	3 months
S. mutans $1x10^7$ cfu/mL	7.5	7.1	<1	<1	<1	<1
L. casei $1x10^9$ cfu/mL	8.3	8.4	<1	<1	<1	<1
A. viscosus $1x10^6$ cfu/mL	6.5	6.4	<1	<1	<1	<1
N. mucosa $1x10^4$ cfu/mL	6.3	<1	<1	<1	<1	<1
V. dispar $1x10^9$ cfu/mL	8.7	8.8	<1	<1	<1	<1
P. melanogenicus $1x10^7$ cfu/mL	7.6	7.5	<1	<1	<1	<1
F. nucleatum $1x10^8$ cfu/mL	8.3	8.2	<1	<1	<1	<1
S. mitis $1x10^{10}$ cfu/mL	8.4	8.4	5.3	<1	<1	<1
S. sanguinis 1×10^8 cfu/mL	7.4	7.1	5.1	<1	<1	<1

The results show that after 1 week storage at -80°C in 5% v/v glycerol, there was a marked reduction in viability of all species. Therefore, it can be concluded that 5% v/v glycerol is not able to provide a suitable shelf-life that would add any benefit to the model system.

The results in Table 8 show the recovery of the organisms in 10% glycerol held at -80°C for up to three months in 2 mL Eppendorf tubes.

Table 8 Stability of individual organisms held in 10% glycerol for 3 months at -80°C, reported in Log_{10}

Organisms (starting concentration)	**Initial (post-dilution)**	**1 week**	**2 weeks**	**1 month**	**2 months**	**3 months**
S. mutans $1x10^7$ cfu/mL	7.5	7.5	7.4	7.4	7.3	<1
L. casei $1x10^9$ cfu/mL	8.3	8.4	8.4	8.4	6.0	<1
A. viscosus $1x10^6$ cfu/mL	6.5	6.5	6.5	6.4	5.4	<1
N. mucosa $1x10^4$ cfu/mL	6.3	6.2	6.4	6.3	<1	<1
V. dispar $1x10^9$ cfu/mL	8.7	8.8	8.7	8.6	<1	<1
P. melanogenicus $1x10^7$ cfu/mL	7.6	7.5	7.5	7.5	5.1	<1
F. nucleatum $1x10^8$ cfu/mL	8.3	8.3	8.3	8.3	<1	<1
S. mitis $1x10^{10}$ cfu/mL	8.4	8.4	8.4	8.4	7.5	<1
S. sanguinis $1x10^8$ cfu/mL	7.4	7.6	7.5	7.4	7.3	<1

The results from Table 7 and Table 8 both show bacterial viability can be retained after -80°C storage. Ten per cent v/v glycerol showed the better recovery; however, even at this concentration viability was lost after two months storage. Therefore, it is recommended that the defined inoculum be stored at 10% v/v glycerol for 1 month at -80°C. This would be a considerable time saving to the running of the methodology. Investigating 15% v/v glycerol, may help extend this further, however this was not carried out at this time.

4.3.4 Stability Testing of Individual Inoculum in HA-Coated Microtitre Plates

The results in Table 9 show the results for individual inoculum held at -80°C in 5% glycerol for up to three months in the HA coated microtitre plates.

Table 9 Stability of individual organisms held in a HA-coated microtitre plate in 5% glycerol for 3 months at -80°C, reported in Log_{10}

Organisms (starting concentration)	Initial (post-dilution)	1 week	2 weeks	1 month	2 months	3 months
S. mutans $1x10^7$ cfu/mL	7.5	<1	<1	<1	<1	<1
L. casei $1x10^9$ cfu/mL	9.3	<1	<1	<1	<1	<1
A. viscosus $1x10^6$ cfu/mL	6.4	<1	<1	<1	<1	<1
N. mucosa $1x10^4$ cfu/mL	4.5	<1	<1	<1	<1	<1
V. dispar $1x10^9$ cfu/mL	9.3	<1	<1	<1	<1	<1
P. melanogenicus $1x10^7$ cfu/mL	7.6	<1	<1	<1	<1	<1
F. nucleatum $1x10^8$ cfu/mL	8.3	<1	<1	<1	<1	<1
S. mitis $1x10^{10}$ cfu/mL	9.4	<1	<1	<1	<1	<1
S. sanguinis 1x108 cfu/mL	8.3	<1	<1	<1	<1	<1

Unfortunately, the results show again that 5% v/v glycerol is not suitable for preserving the inoculum during storage at -80°C on a HA coated microtitre plate.

Table 10 Stability of individual organisms held in a HA-coated microtitre plate in 10% glycerol for 3 months at -80°C, reported in Log_{10}

Organisms (starting concentration)	Initial (post-dilution)	1 week	2 weeks	1 month	2 months	3 months
S. mutans $1x10^7$ cfu/mL	7.5	<1	<1	<1	<1	<1
L. casei $1x10^9$ cfu/mL	9.3	<1	<1	<1	<1	<1
A. viscosus $1x10^6$ cfu/mL	6.4	<1	<1	<1	<1	<1
N. mucosa $1x10^4$ cfu/mL	4.5	<1	<1	<1	<1	<1
V. dispar $1x10^9$ cfu/mL	9.3	<1	<1	<1	<1	<1
P. melanogenicus $1x10^7$ cfu/mL	7.6	<1	<1	<1	<1	<1
F. nucleatum $1x10^8$ cfu/mL	8.3	<1	<1	<1	<1	<1
S. mitis $1x10^{10}$ cfu/mL	9.4	<1	<1	<1	<1	<1
S sanguinis $1x10^8$ cfu/mL	8.3	<1	<1	<1	<1	<1

Again, it was seen that in the HA coated microtitre plate, the inoculum did not remain viable under starage at -80°C. In comparison with storage in the 2 mL Eppendorf tubes (Table 8), there is no retention of activity, even with the 10% v/v glycerol.

Thus, it is recommended that the defined inoculum be stored at in 10% v/v glycerol in 2 mL Eppendorf tubes for a maximum of one month to ensure viability of all species is retained.

4.3.5 Correlation of MFU with Planktonic CFU

Below are the results comparing cfu/mL with MFU and absorbance in

Table 11.

Table 11 Correlation of absorbance at 600 nm with CFU in BHI and BHI + 10% glycerol broth.

		BHI			**BHI+10% Glycerol**		
		Ab@ 600nm	**Log_{10} CFU**	**MFU Count**	**Ab@ 600nm**	**Log_{10} CFU**	**MFC Count**
S. mutans Inoc = $2.3x10^8$ cfu/mL	Base	0.000	----------	429	0.000	--------	459
	1 hr	0.003	6.07	366	0.006	6.24	545
	2 hrs	0.004	6.03	510	0.003	6.13	672
	3 hrs	0.006	7.28	361	0.003	6.18	524
	4 hrs	0.009	6.23	691	0.003	6.68	934
	5 hrs	---------	----------	-------	--------	--------	-------
	6 hr	0.016	6.47	540	0.005	6.26	380
	7 hrs	0.023	7.08	903	0.006	6.33	524
	24 hrs	0.338	11.72	5172	0.713	11.74	4872
	48 hr	0.603	11.21	3281	0.798	11.65	1174
L. casei Inoc. = $2.1x10^9$ cfu/mL	Base	0.000	----------	-------	0.000	--------	-------
	2 hrs	0.005	7.50	452	0.003	6.59	508
	4 hrs	0.009	7.47	585	0.006	6.51	578
	6 hrs	0.021	6.65	602	0.011	6.86	844
	24 hrs	0.554	9.48	4640	0.199	10.49	2781
	48 hrs	0.511	10.76	4470	0.255	7.77	3265
N. mucosa Inoc.= $4.5x10^5$ cfu/mL	Base	0.000	--------	-------	0.000	-------	-------
	2 hrs	0.010	4.44	457	0.001	4.83	530
	4 hrs	0.010	5.28	475	0.004	4.24	553
	6 hrs	0.010	6.51	466	0.005	3.57	503
	24 hrs	1.332	9.17	4503	0.070	9.70	939
	48 hrs	1.150	8.41	4145	0.627	11.30	4396
S.mitis Inoc. = 1856 cfu/mL	Base	0.000	---------	-------	0.000	------	------
	2 hrs	0.022	3.26	230	0.011	2.99	252
	4 hrs	0.031	4.60	443	0.021	4.67	493
	6 hrs	0.034	2.94	439	0.025	2.16	491
	24 hrs	0.462	7.31	538	0.400	4.51	293
	48 hrs	0.561	6.89	4014	0.443	6.69	1843
S.sanguinis Inoc. = $2.27x10^4$ cfu/mL	Base	0.000	---------	-------	0.000	-------	-------
	2 hrs	0.124	4.67	233	0.003	4.62	244
	4 hrs	0.139	3.15	443	0.007	4.42	494
	6 hrs	0.141	8.04	439	0.010	4.56	496
	24 hrs	1.188	9.32	538	0.763	8.62	3818
	48 hrs	1.182	7.30	4080	0.748	9.4	3600
F. nucleatum Inoc. =$2.00x10^4$ cfu/mL	Base	0.000	---------	--------	0.000	-------	-------
	2 hrs	0.002	3.33	462	0.001	3.40	956
	4 hrs	0.004	3.98	884	0.003	3.51	2363
	6 hrs	0.005	2.31	542	0.005	3.31	1336

	24 hrs	0.012	3.30	590	0.010	3.31	1551
	48 hrs	0.013	1.20	415	0.010	1.30	657
P. melaninogenicus Inoc. = $9.4x10^3$ cfu/mL	Base	0.000	---------	--------	0.000	--------	-------
	2 hrs	0.002	8.66	557	0.002	8.51	1576
	4 hrs	0.006	8.56	936	0.001	8.67	3011
	6 hrs	0.007	8.22	576	0.009	8.54	2165
	24 hrs	0.021	8.12	428	0.019	6.66	1912
	48 hrs	0.191	4.54	428	0.020	3.59	765
A. viscosus Inoc. = $2.64x10^9$ cfu/mL	Base	0.000	--------	--------	0.000	-------	-------
	2 hrs	0.001	8.66	391	0.002	8.51	353
	4 hrs	0.003	8.56	455	0.001	8.67	404
	6 hrs	0.010	8.22	859	0.009	8.54	662
	24 hrs	0.174	8.12	1334	0.019	6.66	854
	48 hrs	0.191	4.54	670	0.020	3.59	543
V. dispar Inoc.= $5.28x10^9$ cfu/mL	Base	0.000	--------	-------	0.000	-------	-------
	2 hrs	0.004	9.22	423	0.001	9.52	347
	4 hrs	0.005	9.13	472	0.003	9.31	401
	6 hrs	0.013	10.06	859	0.007	8.48	662
	24 hrs	0.152	9.99	1618	0.025	7.30	707
	48 hrs	0.163	8.91	684	0.031	6.19	433

The relationship analysis looked at the change in ratio over time verses Log_{10} cfu as this is the known variable, and MFU and Ab as the unknown variables. All the results were graphically represented below, however, between 6-7 hour measurement and 24 hours, some of the relationships were compromised. It is hypothesied that this was due to the rate limiting step of available nutrients. Therefore, to fully understand any relationships, the results will be graphically represented from1 to 6-7 hours.

In this analysis R^2 was calculated where R_2= correlation coefficient where 1= perfect correlation. For this analysis $R^2 < 0.98$ was not deemed to be a close correlation. $R^2 > 0.98$ was deemed to be a close correlation where the results could be utilised to determine MFU, Ab or cfu where one parameter was already known.

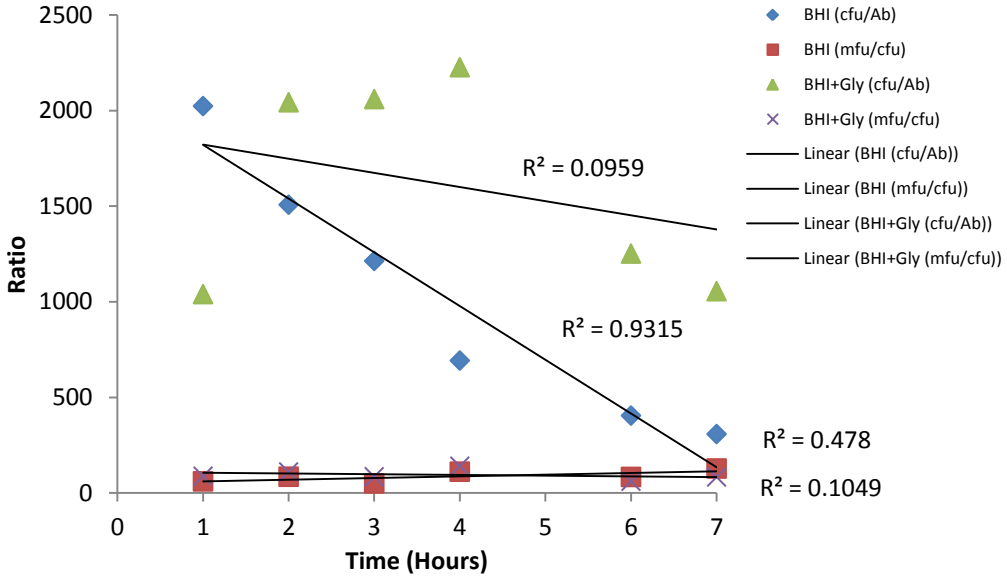


Figure 40 *Streptococcus mutans* relationship analaysis**.** The results show poor correlation where $R^2 < 0.98$, where no relationship could be identified between MFU, cfu or Ab.

The results from Figure 40 show that no correlation could be identified between cfu, MFU and absorbance measurements.

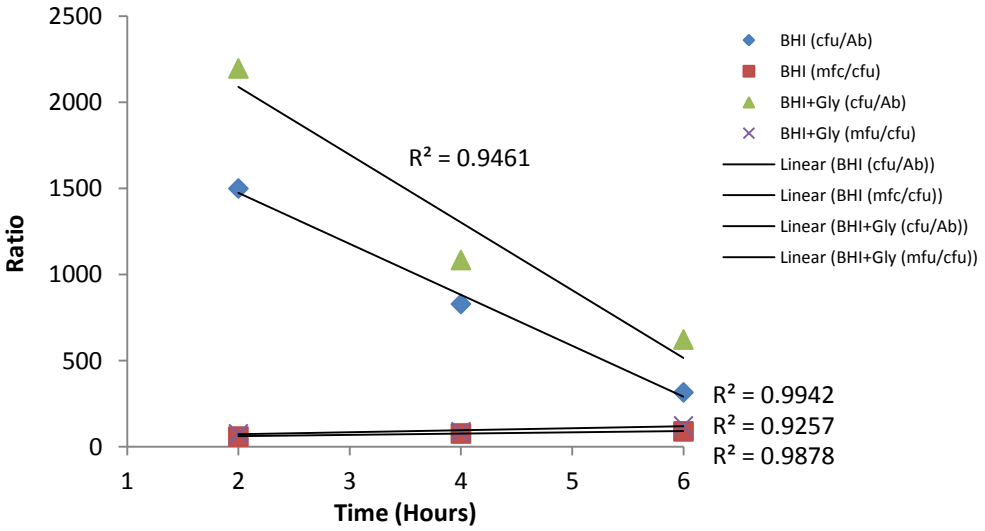


Figure 41 *Lactobacillus casei* relationship analysis.The results show poor correlation where $R^2 < 0.98$ for the organisms grown in BHI + 10% v/v glycerol. A good correlation was identified between cfu, MFU and absorbance for organisms grown in BHI

Figure 41 shows a poor correlation between cfu, absorbance and MFU for *L. casei* grown in BHI + 10% v/v glycerol. However, a good correlation was found where *L. casei* was grown in BHI. Therefore, for up to 6 hours post-incubation, where one parameter is known, using the ratio, the other parameters can be determined.

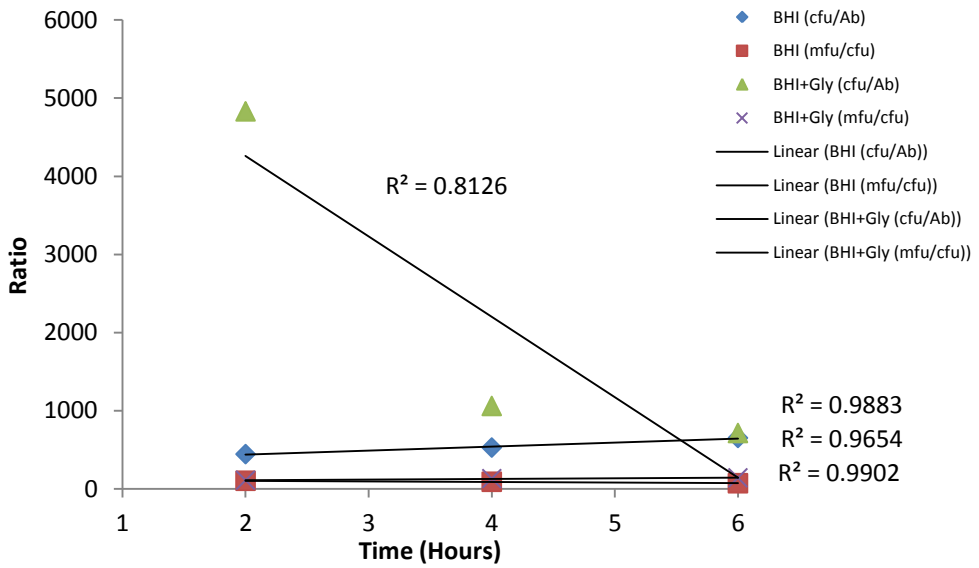


Figure 42 *Neisseria mucosa* relationship analysis. The results show poor correlation where R^2<0.98 for the organisms grown in BHI + 10% v/v glycerol. A good correlations was identified between cfu, MFU and absorbance for organisms grown in BHI.

Figure 42 shows a poor correlation between cfu, MFU and absorbance for *N. mucosa* grown in BHI + 10% v/v glycerol. However, a good correlation was found where *N. mucosa* was grown in BHI. Therefore, for up to 6 hours post-incubation, where one parameter is known, using the ratio, the other parameters can be determined.

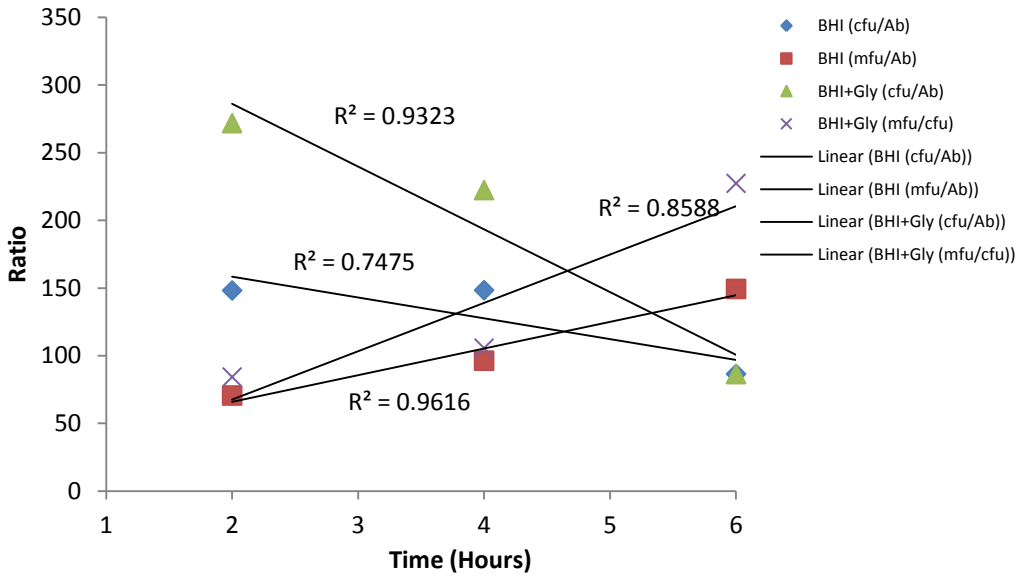


Figure 43 *Streptococcus mitis* relationship analysis. The results show poor correlations where R^2<0.98, where no relationship could be identified between MFU, cfu or Ab

Figure 43 showed that no correlation could be identified between cfu, MFU or absorbance for *S. mitis*.

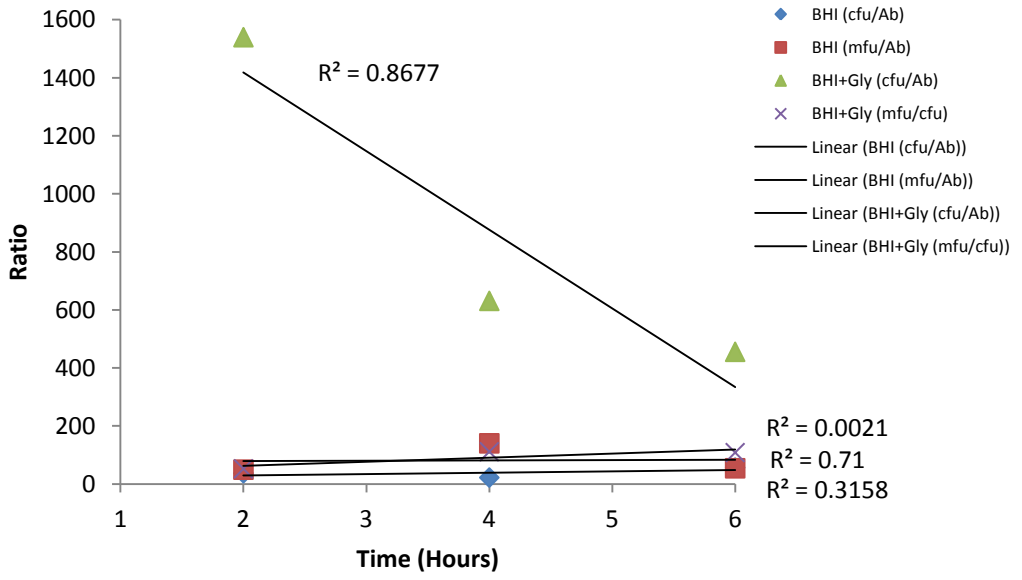


Figure 44 *Streptococcus sanguinis* relationship analysis. The results show poor correlation where R^2<0.98, where no relationship could be identified between MFU, cfu or Ab.

Figure 44 show that no correlation could be identified for *S. sanguinis* between cfu, MFU and absorbance.

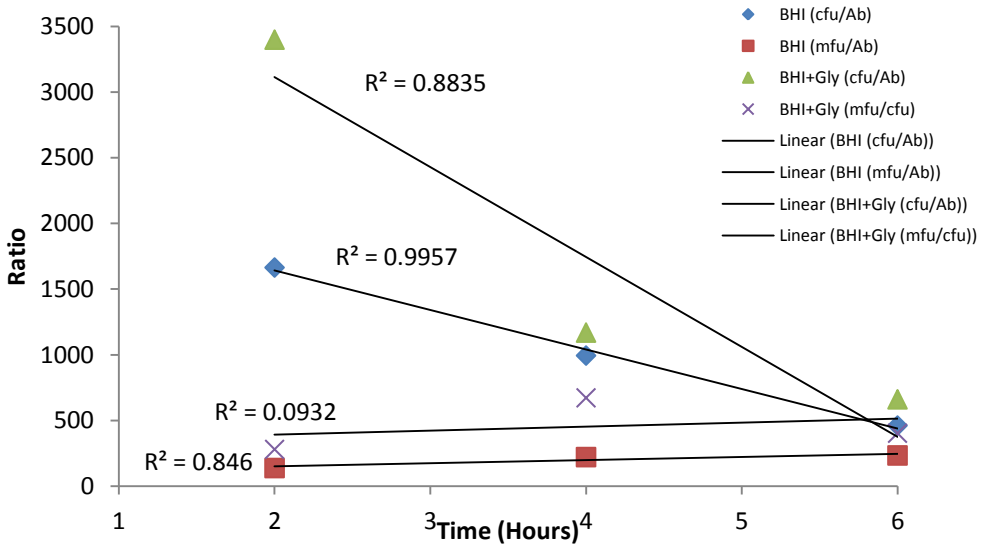


Figure 45 *Fusobacterium nucleatum* relationship analysis. The results show poor correlation where R^2<0.98, however a good correlation was identified when the organism is grown in BHI, between cfu and absorbance where R=0.9957.

Figure 45 show that only one correlation could be identified for F. nucleatum and the relationship between cfu and absorbance. No other correlations were identified using this species between MFU, cfu and absorbance, where, in all instances R^2<0.98.

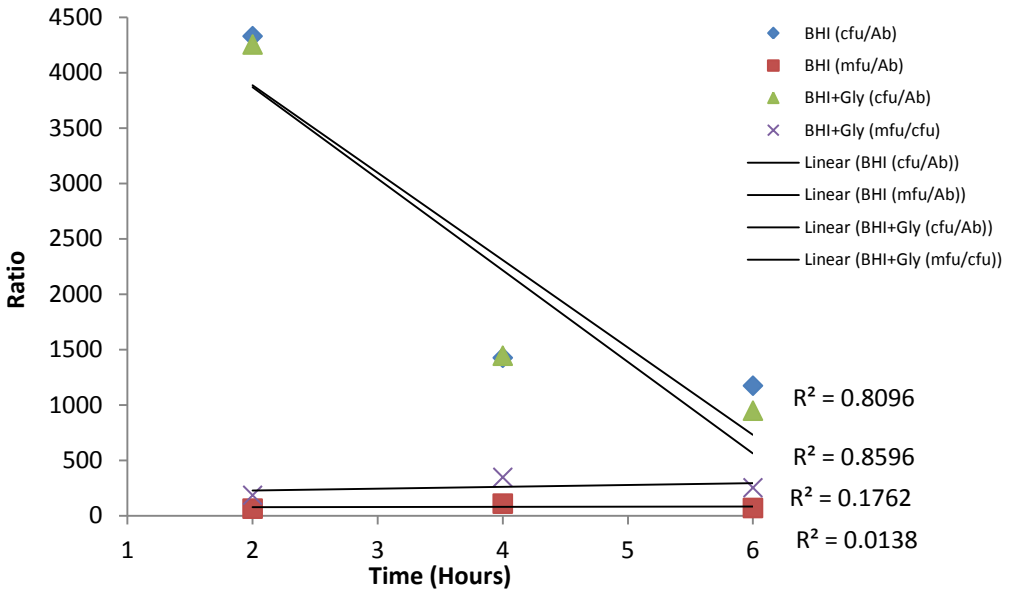


Figure 46 *Prevotella melaninogenicus* relationship analysis. The results show poor correlation between cfu, MFU and absorbance where in all instances R^2<0.98.

Figure 46 shows no correlation could be identifeied for P. melaninogenicus between cfu, MFU and absorbance.

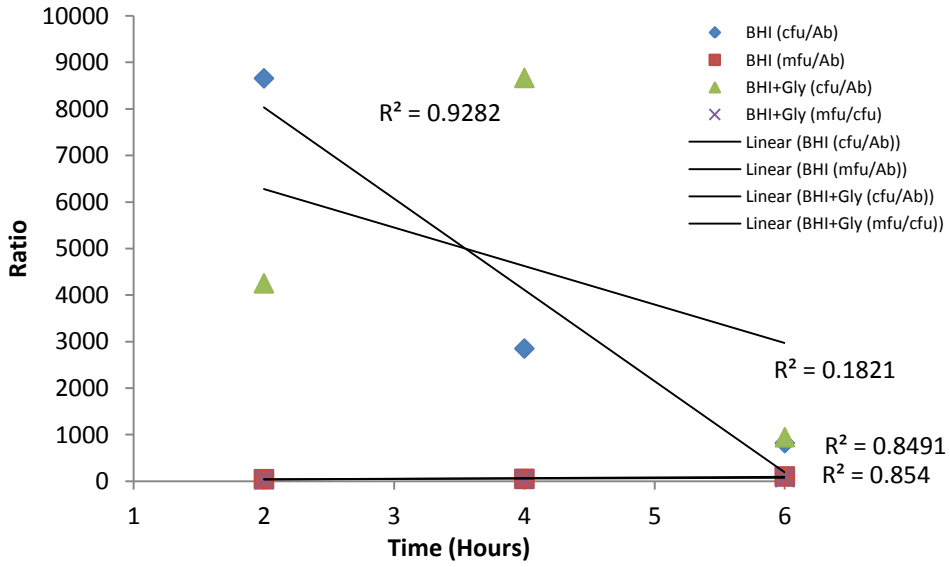


Figure 47 *Actinomyces viscosus* relationship analysis. The results show poor correlation between cfu, MFU and absorbance where R^2<0.98.

The results from Figure 47 shows that no correlation could be identified for *A. viscosus* between cfu, MFU and absorbance.

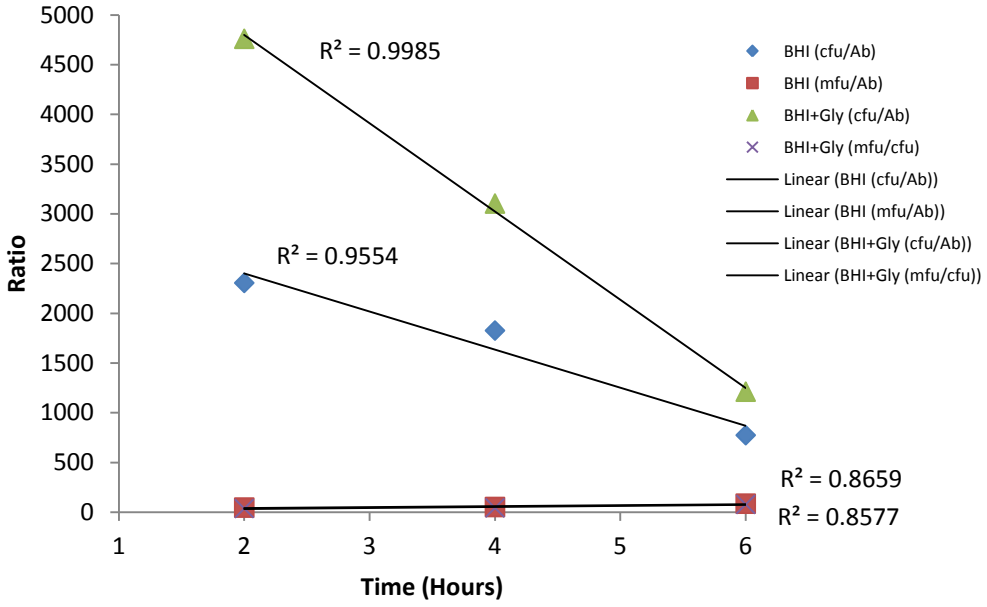


Figure 48 *Veillonella dipar* relationship analysis. The results show good correlation where R^2=0.9985 for the organisms grown in BHI + 10% v/v glycerol (cfu/Ab). For all other results the correlations was poor where R^2<0.98.

Figure 48 shows that only one analysis showed good correlation, where R^2=0.9985. This was where V. dispar was grown in BHI + 10% v/v glycerol and investigated for cfu/Ab correlation. Therefore, for up to 6 hours post-incubation, where one parameter is known (cfu or absorbance), using the ratio, the other parameter can be identified.

4.3.6 Correlation of MFU with Biofilm CFU

From Figure 49 (A and B) is is seen that the repeatability of the CHX dose response is positive, if indeed excellent on the same plate. Figure 49 (B) was further utilised to determine cfu/biofilm. The results can be found in Figure 50 and Table 12.

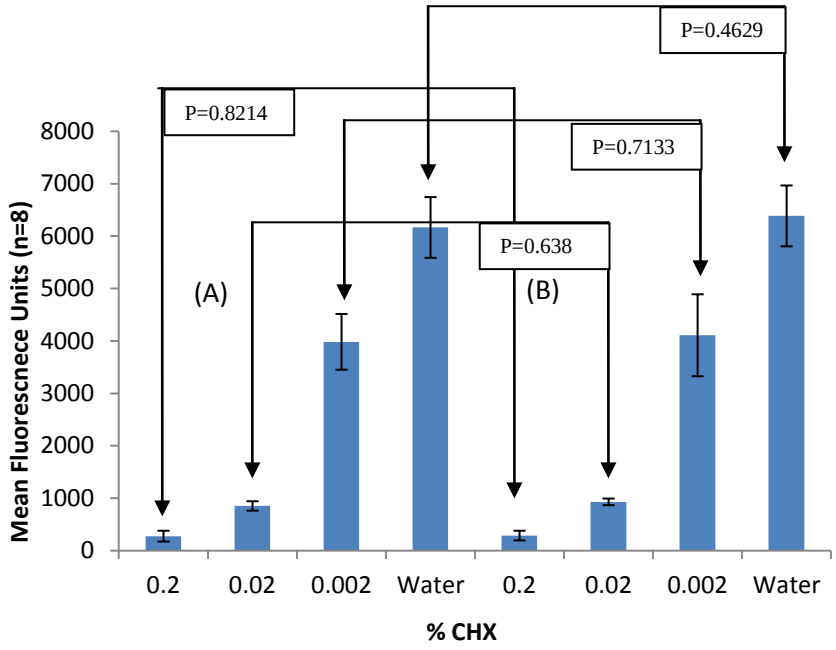


Figure 49 Repeating CHX dose response in the developed Bioplate. No statistical differences between repeat treatments was identified. A continued reduction in viability was seem with an increase in CHX concentration, deionised water was used at a negative control (n=8).

Using One-Way ANOVA between similar test treatments, no statistical differences were found. There is a correlation, in this study that increasing MFU equates to an increase in cfu/biofilm, however, it cannot be extrapolated which organisms are affecting MFU from the results in Figure 50. It should be taken with caution that this result, from Figure 49 can show that MFU and cfu are closely related. The results show, 0.02% v/v CHX to have very low MFU, however, when the organisms are regrown on nutrient agar the cfu is higher than

expected, showing the organisms capacity to recover, as seen in Figure 50. Possibly indicating both bacterial kill and reduction in metabolism due to environmental stresses.

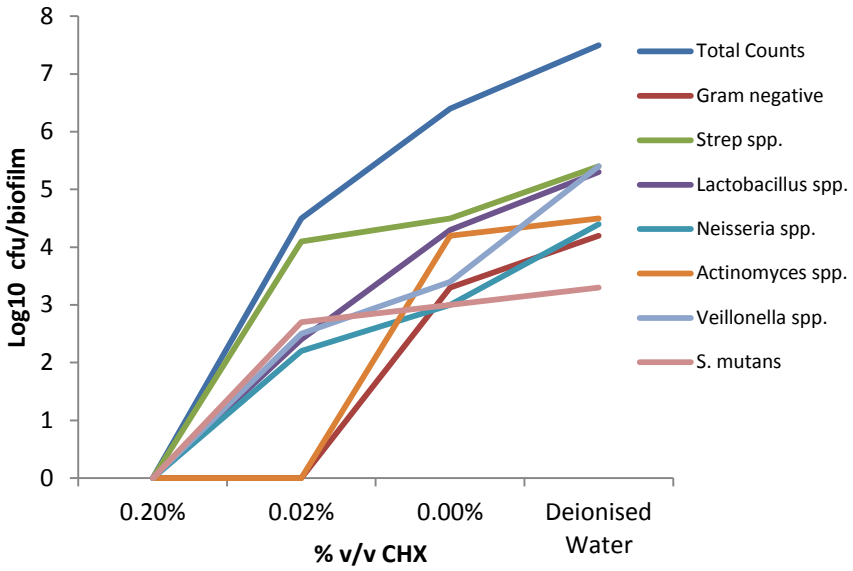


Figure 50 Effect of CHX dose response on bacterial recovery from the Bioplate where n=8. .

Table 12 Effect of CHX dose response on bacterial recovery from the Bioplate

	0.2%	**0.02%**	**0.002%**	**Deionised Water**
Media		**Log_{10} Counts**		
Total Counts	<1	4.5	6.4	7.5
Gram negative	<1	<1	3.3	4.2
Streptococcus spp.	<1	4.1	4.5	5.4
Lactobacillus spp.	<1	2.4	4.3	5.3
Neisseria spp.	<1	2.2	3.0	4.4
Actinomyces spp.	<1	<1	4.2	4.5
Veillonella spp.	<1	2.5	3.4	5.4
S. mutans	<1	2.7	3.0	3.3

4.4 Discussion

This section investigated the possibility of improving the model system by investigating the stability of the bioplate, therefore, offering a truly 'off-the-shelf' biofilm model system. The key finding in this chapter was the utilisation of mixed species inocula held at -80°C for one month in 10% glycerol. This permits a faster turnaround for testing, as the initial bacteria growth and set up has been removed. This is a saving of 7 days set up before each analysis. None of the analyses utilising 5% glycerol, showed any retention of bacterial cell viability beyond 2 weeks.

It should be noted, it was anticipated that the organisms would remain viable on the HA coated microtitre plates, in 10% glycerol after storage at -80°C for at least 1 month. This however was not the case, where a reduction in viability was seen with a number of species at this time point. It is hypothesised that this may be due to the very small volume placed in each of the wells, and the large surface area created via the HA coating. A review of the literature shows no research into the effect of small volume storage, with respect to surface area, to support or contradict this hypothesis and furthermore, further work would need to be carried out to support this hypothesis.

It is also recommended the following concentrations be generated following that recommended by McKee (1985), *S. mutans* ($1x10^7$ cfu/mL), *S. sanguinis* ($1x10^8$ cfu/mL), *S. mitis* ($1x10^{10}$ cfu/mL), *A. viscosus* ($1x10^6$ cfu/mL), *L. casei* ($1x10^9$ cfu/mL), *N. mucosa* var. *mucosa* ($1x10^6$ cfu/mL), *V. dispar* ($1x10^9$ cfu/mL), *P.*

melaninogenica ($1x10^7$ cfu/mL) and *F. nucleatum* ($1x10^8$ cfu/mL) for use as the defined inoculum. One hundred microlitres of each organism should be added to a 2 mL Eppendorf tube and centrifuged at 8000 rpm for 10 minutes and the supernatant removed. This should then be replaced with 900 µL of 10% v/v glycerol in R+P and store at -80°C for 1 month.

As stated in the introduction, Mariscal *et al.* (2009) utilised resazurin dye as an indicator of bacterial respiration. In this study correlation with bacterial numbers was also investigated and showed that it is possible to estimate bacterial counts via the generation of initial growth curves. It should be noted that this method was recommended if the bacterial numbers were between $1x10^3$ – $1x10^8$ CFU for an estimate of the CFU to be made after 5 hours. However, this study utilised single species. The biofilm model developed as part of this PhD utilised mixed species biofilms to be more representative of what would be found in the oral cavity. These differences in growth rates and respiration of these different organisms make this method of correlation between cell count and MFU unreliable in this biofilm model.

In the next chapter, I aim to investigate other peer reviewed biofilm model systems for the development or orally relevant biofilms, and their ability to rank and screen antimicrobial actives.

CHAPTER 5: Comparison of Bioplate model with peer reviewed *in vitro* model systems

5.1 Introduction

The search for new antimicrobial agents for improved biofilm control within the oral cavity requires that appropriate screening models be put in place. Key criteria for these models would ideally include; 1. The ability to be predictive of clinical outcome, 2. The use of orally relevant organisms (mixed species), 3. Bacteria present in biofilms, 4. Short contact time relevant to the use of oral care products, 5. Reproducibility and 6. High-throughput.

In situ oral biofilm models are considered the 'gold standard' of oral biofilm research as they permit the generation and testing of biofilms under their native environments, reflecting what would be seen clinically in the absence of host factors. A number of *in situ* models have been developed to investigate caries (Gameiro *et al.,* 2009; Zero, 1995), dentinal caries (Lima *et al.,* 2009), fluoride penetration through plaque (Robertson *et al.,* 1997; Watson *et al.,* 2004; Watson *et al*., 2005) and to monitor the effect of preservatives on oral biofilms (Arweiler *et al.,* 2008). However, these studies tend to very expensive and time consuming to run as screening models.

In vitro model systems offer controlled systems for the evaluation of biofilms. There are a number of peer reviewed systems currently available, CDFF, MBEC and Sorbarod for modelling biofilms found in the oral cavity, and subsequent testing of antimicrobial agents against these biofilms.

The constant depth film fermenter (CDFF) concept was originally discussed in 1974 by Atkinson and Fowler, focussing primarily on a microbial film fermenter. Utilising this concept and building on research carried out by Coombe (1981), further model developments by Peters and Wimpenny in 1988, resulted in a model that would allow the development of replicate biofilms of a defined thickness. This model permits the development of biofilms between 50-600 μm thick due to the biofilms being grown in recessed pans. The thickness is limited by a static Teflon blade which sweeps excess biomaterial from the pans as the turntable rotates. This is a closed model system where the environmental gas, medium and inoculum can be controlled. The biofilms can be removed and evaluated for bacterial counts, architecture and antimicrobial efficacy of any test substances. The CDFF model system has been widely used in oral biofilm development and research (McBain *et al.,* 2003; Metcalf *et al.,* 2006; Pratten *et al.,* 1998; Vroom *et al.,* 1999; Wilson, 1990).

The MBEC model was developed from the Calgary Biofilm Device (CBD) and developed by Ceri *et al.,* (1999). A key reason for utilising this methodology is that it is a peer reviewed, 96-well microtitre plate biofilm screening model. The MBEC model system utilises both the lid, which contains 96 pegs, corresponding to each of the 96-wells on the base of the plate. Once a test inoculum is added to the microtitre plate and the plate incubated at an appropriate temperature and time, biofilms will form on the pegs. The model was developed to investigate the antibiotic susceptibility of attached bacteria, whilst in a biofilm state.

Previous studies have demonstrated that the perfused Sorbarod biofilm system can maintain stable bacterial communities (Ledder *et al.,* 2006; McBain *et al*., 2005). Orally generated malodour can be controlled by a number of methods such as masking, inactivation/neutralisation of malodourous molecules or use of antimicrobial agents to kill the volatile sulphur compound (VSC) producing organisms (Carvalho *et al.,* 2004; Young *et al.,* 2003). The Sorbarod biofilm permits the development of an orally relevant mixed species biofilm model in which test actives can be evaluated for their impact on VSC levels and antibacterial ability under conditions of flow. The model is based on the Perfused Biofilm Model (Hodgson *et al.*, 1995), modified to include an orally relevant malodourous biofilm derived from tongue dorsum scrapings (Greenman *et al.*, 2005), and uses a Sorbarod filter (cylindrical paper sleeve encasing a compacted concertina of cellulose fibres, like a cigarette filter) to allow the attachment and growth of bacteria. Continuous flow of media provides a substantivity challenge when evaluating the efficacy of test agents. The model was used to compare the anti-VSC efficacy of various agents with antimicrobial and/or VSC neutralising activity employing gas chromatograph analysis for the determination of hydrogen sulfide and methyl mercaptan concentrations, building on a model designed by Spencer *et al*. (2007) utilising a Halimeter to monitor total VSC's.

5.2 Materials and Methods

5.2.1 Constant Depth Film Fermenter

The CDFF employed in this study was a modified version first developed by Peters and Wimpenny in 1988. An overview of the model is discussed in chapter 2.

5.2.1.1 Inoculation of the Constant Depth Film Fermenter

A defined inoculum was used to inoculate the CDFF, and was plated out on relevant media (full details can be found in chapter 4) to obtain required concentrations. The organisms used were, *S. mutans* NCTC 10449 (10^8 cfu/mL), *S. mitis* NCTC 12261 (10^{10} cfu/mL), *S. sanguinis* NCTC 10904 (10^6 cfu/mL), *A. viscosus* NCTC 10951 (10^6 cfu/mL), *L. casei* var. *rhamnosus* NCTC 10302 (10^{10} cfu/mL), *N. mucosa* NCTC 10774 (10^9 cfu/mL), *V. dispar* NCTC 11831 (10^5 cfu/mL), *P. melaninogenica* NCTC 11321 (10^9 cfu/mL) and *F. nucleatum* NCTC 10562 (10^5 cfu/mL). One millilitre of each organism; *S. mutans, S. mitis, S. sanguinis* and *L. casei* was added along with 4 mL of *A. viscosus, N. mucosa, V. dispar, P. melaninogenica, F. nucleatum* to 300 mL of artificial saliva. The artificial saliva was prepared by mixing:

- 0.7 g Lab-lemco powder (Oxoid, UK),
- 0.14 g yeast extract (Oxoid, UK),
- 3.5 g proteose peptone (Oxoid, UK),
- 1.75 g hog gastric mucin (Sigma, UK),
- 0.245 g sodium chloride (Sigma, UK),

- 0.14 g calcium chloride (Sigma, UK),
- 0.14 g potassium chloride (Sigma, UK)
- 300 mL deionised water

and autoclaving at 121°C for 15 minutes. When cooled 0.875 mL of 40% w/v urea (Oxoid, UK) was aseptically added to the medium. This is based on the artificial saliva developed by Russell and Coulter (1975).

The inoculum was added to the CDFF for 5 hours at a rate of 0.5 mL/min, incubated at 37°C aerobically and continually mixed with a magnetic stirrer. This was equivalent to the mean salivary flow rate of 0.72 litres/day for humans (Guyton, 1992). Subsequently, the inoculum was removed and the CDFF was then continually fed with artificial saliva at a rate of 0.34 mL/min. The CDFF unit was incubated at 37°C aerobically. At various time points biofilms were removed for analysis.

5.2.1.2 Susceptibility Testing of the Constant Depth Film Fermenter

At defined time points (1, 3, 5 and 8 days) a PTFE pan with associated biofilm was removed from the CDFF using the procedure above. Two of the biofilms were aseptically removed from the PTFE pan and each placed in 10 mL sterile R+P individually. This was then vortexed for 1 minute to disrupt the biofilms. Serial dilutions were carried out in 9 mL of R+P from 10^{-2} to 10^{-10} dilution on selective and non-selective agars in duplicate (as noted in Table 13). This gave baseline counts for the biofilms samples at each time point.

Table 13 Selective and non-selective agars

Organism	**Media**
Total Counts	6% v/v Horse blood agar
Gram-negative	6% v/v Horse blood agar + 2.5 µg/mL vancomycin, incubate anaerobically
Streptococcus spp.	Mitis salivarius agar (BD, Franklin Lakes, USA)
Lactobacilli spp.	Rogosa agar (Fluka)
S. mutans	6% v/v Horse blood agar + 100 µg/mL streptomycin
Neisseria spp.	6% v/v Horse blood agar + 2.5 µg/mL vancomycin incubate aerobically
Actinomyces spp.	*Actinomyces* Isolation Media (Zylber and Jordan, 1982)
Fusobacterium spp.	*Fusobacterium* media
Veillonella spp.	*Veillonella* agar

Agars were prepared using the following methods, for 6% v/v horse blood agar, 42.5 g blood agar base No 2 (Sigma), and boiled in 1 L of deionised water to dissolve, a magnetic flea was added to the container, and autoclaved at 121°C for 15 minutes. Once cooled to 45-55°C, 60 mL of defibrinated horse blood (Southern Group Laboratories, Corby, UK) was aseptically added and mixed. Agar was pour plated into 90 mm Petri dishes, expiry dated for two weeks and stored between 2-8°C until use. Following the above method to prepare 6 % horse blood, to make the gram-negative media, 0.25 g of vancomycin (Sigma) was added to 100 mL of sterile deionised water and mixed to dissolve. One mL of this was added to 1 L of the prepared horse blood media to generate an in-use concentration of 2.5 µg/mL at the same time as addition of the horse blood. For the recovery of *S. mutans* the same method was followed with respect to the preparation of 6% v/v horse blood agar, to generate 100 µg/mL final concentration of streptomycin, 1 g of streptomycin (Sigma) was added to 10 mL of sterile deionised water, and mixed to dissolve. One millilitre was subsequently

added to the autoclaved media once cooled to between 45-55°C. Rogosa agar was manufactured following the manufacturers (Fluka) instructions, 73 g of the dehydrated media was added to 1 L and boiled to dissolve, one vial of Tween 80 (Fluka) was added and the media filled into 90 mm Petri dishes. This media is not autoclaved. *Actinomyces* isolation media was based on the media developed by Zylber and Jordan (1982), and was purchased pre-prepared from Anaerobe Systems (Morgan Hill, CA, USA). Mitis salivarius agar (BD, Franklin Lakes, NJ, USA) was prepared following the manufacturer's instructions, 90 g of the dehydrated media was added to 1 L deionised water and a magnetic flea added to the mixture, boiled to dissolve and autoclaved at 121°C for 15 minutes. Once cooled, 1 mL of 1% Tellurite solution (BD) was aseptically added and mixed. *Fusobacterium* agar was purchased pre-prepared from Anaerobe systems. *Veillonella* media was prepared as follows, agar (Oxoid), 15 g; tryptone (Sigma), 5 g; yeast extract (Oxoid), sodium thioglycollate (Sigma), 0.75 mL, basic fushcin (Sigma), 0.002 g; sodium lactate (VWR), 2.4 mL was added to 1 L deionised water. Media was autoclaved at 121°C for 15 minutes and once cooled, 1 mL of 5 mg/mL vancomycin (Bioconnections, Weatherby, UK) was added and mixed.

5.2.1.3 Antimicrobial Testing of Developed Biofilms

As found in the previous study to reach a steady biofilm state took 5 days post-inoculation. Two biofilms were removed after 5 days incubation from a pan, and placed in 10 mL R+P individually and vortexed for one minute to remove the biofilm from the HA substrate. Serial dilutions were carried out in 9 mL R+P

from 10^{-2} to 10^{-10} dilutions on selective and non-selective agars in duplicate as described in 5.2.1.5. This gave initial counts prior to testing.

Subsequently, after 5 days a pan was removed and placed in a 60 mL Sterilin pot containing either 0.5, 0.1 or 0.1% v/v CHX or a 60 mL Sterilin pot containing either 1 or 0.5% triclosan, taking care not to disturb the intact biofilms. After two minutes the pans were removed and gently washed in sterile R+P, again, taking care not to disrupt the biofilms. Subsequently, two biofilms were removed and placed in 10 mL R+P and vortexed for 1 minute to remove the biofilm from the HA substrate. Serial dilutions were carried out in 9 mL R+P from 10^{-2} to 10^{-10} dilutions on selective and non-selective agars in duplicate as described in 5.2.1.2.

5.2.2 Minimum Biofilm Eradication Concentration (MBEC)

The original model developed was called the Calgary Biofilm Device (CDB) and was developed by Ceri *et al*. (1999). A key reason for utilising this methodology is that it is a peer reviewed, 96-well microtitre plate biofilm screening model. An overview of the model can be found in chapter 2.

5.2.2.1 Growth of Bacterial Subcultures

Utilising the stock organisms held on either TSA or 6% blood agar slopes, *S. mitis* NCTC 12261, *A. viscosus* NCTC 10951, *L. casei* var. *rhamnosus* NCTC 10302, *N. mucosa* var. *mucosa* NCTC 10774, *V. dispar* NCTC 11831, *P. melaninogenica* NCTC 11321 and *F. nucleatum* NCTC 10562 were sub-cultured onto appropriate

agar, either TSA or 6% blood agar to obtain enough viable organisms, and to confirm purity of sub-culture to be utilised in these studies. Sub-cultures were not grown on agars containing selective agent, some antibiotics can initiate an adaptive response in the culture and this may result in modified biofilms. Each organism was subsequently suspended in R+P diluent, suspension was adjusted to 1.0 McFarland standard. One millilitre of *S. mutans*, *S. sanguinis* and *S. mitis*, was placed in 29 mL BHI broth, *A. viscosus*, *L. casei*, *N. mucosa* var. *mucosa*, *V. dispar*, *P. melaninogenica* and *F. nucleatum* were placed in a modified BHI with 0.0001% w/v haemin (Sigma, UK). Haemin stock solution (0.2% w/v) was manufactured by first adding 200 mg of haemin in 50 mL 1M potassium hydroxide solution and adding 50 mL ethanol. All standard inocula were serially diluted and plated to determine the initial bacterial concentrations.

Defined inoculum was used to inoculate the MBEC model using the same inocula generated in 5.2.1.2 using the method in chapter 4. To summarise, the organisms used were *S. mutans* NCTC 10449 (10^8 cfu/mL), *S. mitis* NCTC 12261 (10^{10} cfu/mL), *S.sanguinis* NCTC 10904 (10^6 cfu/mL), *A. viscosus* NCTC 10951 (10^6 cfu/mL), *L. casei* var *rhamnosus* NCTC 10302 (10^{10} cfu/mL), *N mucosa* NCTC 10774 (10^9 cfu/mL), *V. dispar* NCTC 10831 (10^5 cfu/mL), *P. melaninogenica* NCTC 10321 (10^9) cfu/mL and *F. nucleatum* NCTC 10562 (10^5 cfu/mL). One millilitre of each organism suspension was added to a 30 mL sterile container, to this was added 1 mL of R+P and 10 mL of 20% glycerol in BHI, this was subsequently vortexed to ensure homogeneity. Two millilitres of this inoculum was added to 28 ml BHI and added to the reagent reservoir, ensuring all pegs

reached the growth media. Excess was stored in 2 mL Eppendorfs and stored at -80°C for up to one month.

Opening a MBEC plate, one for each organism, the inocula was added to the reagent reservoir, ensuring all the pegs are placed and reaching the growth media. The plates were then placed on a rocking table in a 37°C incubator. Aerobic organisms were incubated at 37°C for 24 or 48 hours. Organisms which required anaerobic or increased CO_2 environments were incubated without shaking at 37°C for between 1-5 days.

5.2.2.2 Antimicrobial Challenge for MBEC

Described below are details carried out of a dose response of a chlorhexidine, triclosan and zinc chloride. Using a new microtitre plate, sterile deionised water was added to column 1 as a standard control and from column 2-9 was added varying concentrations of CHX including 2, 1, 0.5, 0.25, 0.1, 0.05, 0.01, 0.001% v/v, respectively. Triclosan was solubilised in propylene glycol (5% v/v) and evaluated at 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008 and 0.004% w/v. Propylene glycol (5% v/v) was also used as a negative control. Zinc chloride was dissolved in sterile deionised water and evaluated at 5, 2, 1, 0.8, 0.4, 0.2, 0.1, 0.05, 0.01, 0.001% w/v. In all assays 0.2% v/v CHX and sterile deionised water were used as positive and negative controls respectively.

After removing the inoculated MBEC plates from the incubator, the plates were rinsed in a separate plate prepared previously, which contained R+P buffer

solution. Washing was carried out to remove any unattached or loosely bound organisms. Three pegs were removed from column 12 using sterile forceps and each transferred to 10 mL of R+P diluent. This was subsequently vortexed for 1 minute and serially diluted from 10^{-1} to 10^{-7} and plated in duplicate on appropriate agar, then incubated with respect to the requirements of the individual organism. The remaining pegs, still attached to the lid, were transferred to the 96-well microtitre plates containing the CHX and placed in 37°C incubator for two minutes. Subsequently, the plate was removed from the incubator and rinsed in a separate plate, prepared previously, which contained R+P buffer solution. Two hundred microlitres of 10% AlamarBlue™ in BHI was added to a separate 96-well microtitre plate. The washed pegs were then added to this and re-incubated for 30 minutes at 37°C with shaking. Fluorescence was measured using a microtitre plate reader (Biotek) using a 530 nm excitation filter and a 590 nm emission filter. Results are recorded as mean fluorescence units (MFU).

5.2.3 Sorbarod Biofilm Model

An overview of the model system can be found in chapter 2. This model system was developed to inoculate and investigate tongue derived organisms.

5.2.3.1 Preparation of Inoculum

The inoculum was generated using a sterile toothbrush (Mcleans, GSK, Brentford, UK; autoclaved at 121°C for 15 mins) firmly brushed against the posterior dorsum of the tongue of a single donor and washed in 10 mL of sterile phosphate buffered

saline (PBS). These steps were repeated until an optical density equivalent to a 3.0 McFarlane standard was obtained. The PBS was then added to 10 mL of sterile double strength Brain Heart Infusion (BHI) broth (Oxoid). The inoculum (2 mL) was added to a sterile Sorbarod housed in a 2 mL syringe in the absence of flow. Once inoculated, Sorbarod filters were incubated overnight in an anaerobic cabinet at 37°C, then connected to the gas, media and effluent lines and incubated for a further 48 h with continuous perfusion of gas and media. Previous experiments show that steady state VSC levels were reached after 48 hours incubation of the inoculated Sorbarod under the conditions reported here (data not shown).

5.2.3.2 Evaluation of Test Agents

Chlorhexidine digluconate (0.2% v/v) (Sigma-Aldrich), and two metal salts (0.1% w/v copper (II) gluconate (Sigma-Aldrich) and 0.1% w/v zinc acetate (Sigma-Aldrich) were used to characterise the system. Prior to exposing the tongue biofilms to test agents, peristaltic pumps supplying gas and media were switched off and a gate clip below the Sorbarod was closed to hold the test solutions in contact with the biofilm. Test agents (1 mL) were introduced to the surface of the Sorbarod using a hypodermic needle and. After 1 min contact time, the gate clip was opened and gas and media lines switched on, with the syringe remaining in place. Immediately prior to addition of test agents and at various time points after addition (up to 60 min), 20 mL gas samples were collected and the VSC concentrations determined.

5.2.3.3 Determination of VSC Levels

VSC concentrations were determined by gas chromatography using an Agilent 6890N GC (Agilent Technologies, Stockport, Cheshire, UK) equipped with sulfur specific flame photometric detector (hydrogen 40 mL/min, air 100 mL/min, nitrogen make-up gas 40 mL/min). The system was fitted with a 0.1 mL gas sampling valve held at 120°C. The gas sampling valve and all associated tubing was sulfinert™ treated to minimise loss in VSCs through adsorption to tubing (Thames Restek, UK). The GC was equipped with an 8ft x 1/8-inch Teflon support Chromosil 330 (Supelco, UK) packed column held at 60°C, carrier gas was helium at a flow rate of 45 mL/min. Quantitation of hydrogen sulfide and methyl mercaptan in gas samples was achieved by comparison of peak areas with those obtained from standard hydrogen sulfide and methyl mercaptan gas permeation tubes (Kin Tek Precision Gas Standards Generator, Eco Scientific, Stroud, UK).

5.2.3.4 Scanning Electron Microscopy of Biofilms

Full methodology can be found in chapter 2. The outer sheaths of two Sorbarod filters (one sterile, one containing a 48h old biofilm) were removed and the concertina of fibres opened. Representative samples were mounted on appropriate stubbie holders (Agar Scientific, Stanstead, Cambridgeshire, UK) and imaged uncoated in a Zeiss EVO Scanning Electron Microscope operated at 2.5-3kV (Carl Zeiss SMT Ltd, Cambridge, UK).

5.2.4 Developed Bioplate Model

5.2.4.1 Manufacture of Inocula

Defined inoculum was used to inoculate the Bioplate model using the same inoculum generated in 5.2.1.2 using the method in chapter 4. To summarise, the organisms used were *S. mutans* NCTC 10449 (10^8 cfu/mL), *S. mitis* NCTC 12261 (10^{10} cfu/mL), *S. sanguinis* NCTC 10904 (10^6 cfu/mL), *A. viscosus* NCTC 10951 (10^6 cfu/mL), *L. casei* var *rhamnosus* NCTC 10302 (10^{10} cfu/mL), *N mucosa* NCTC 10774 (10^9 cfu/mL), *V. dispar* NCTC 10831 (10^5 cfu/mL), *P. melaninogenica* NCTC 10321 (10^9 cfu/mL) and *F. nucleatum* NCTC 10562 (10^5 cfu/mL). One millilitre of each organism suspension was added to a 30 mL sterile container, to this was added 1 mL of R+P and 10 mL of 20% glycerol in BHI, this was subsequently vortexed to ensure homogeneity. Twenty microlitres of this inoculum was used to inoculate each well on each of the test wells on a HA coated microtitre plate. Excess was stored in 2 mL Eppendorfs and stored at -80°C for up to one month.

5.2.4.2 Bioplate Testing

Hydroxyapatite coated 96 well microtitre plates were manufactured as in chapter 2 section 2.1.1. To each of the HA coated wells was added 20 µL of the test inocula prepared in section 5.2.1.2. Subsequently, 180 µL of sterile BHI was added to each well. Plates were then incubated aerobically for 24 hours at 37°C on an orbital shaker set at 200 rpm. Full testing methodology can be found in section 2.1.2.

5.2.5 Confocal Laser Scanning Microscopy of Developed Biofilms

Full methodology can be found in section 2.3.2. Biofilms were removed from the CDFF, MBEC or Bioplate and placed in a small cell-culture dish (Bibby Sterilin Ltd, UK) with 10 mL distilled water containing 2 µL of the LIVE/DEAD BacLight™ bacterial viability stain. These were incubated in the dark to allow the stain to develop for 15 minutes and rinsed to remove excess stain. The biofilm structure was examined with an Olympus BX51 microscope to which a Bio-Rad Radiance 2100 laser scanning system and a LUMPlanFI ×40 water lens was incorporated. Two-channel (viable "Live" and nonviable "Dead") confocal image stacks were collected in eight-bit colour depth at a resolution of 1024 x 1024 pixels. The *z*-axis step size was optimized for each image stack, depending upon the total depth of each of the test samples. Images were collated and analysed using Image J software, available at http://rsbweb.nih.gov/ij/.

5.3 Results

5.3.1 Constant Depth Film Fermenter

Using the CDFF, initial investigations were carried out to understand how the biofilm developed overtime with the standardised inoculum. The results below show and increase trend in bacterial recovery post inoculation with the defined inoculum in the CDFF after 1, 3, 5 and 8 days, as seen in Table 14

Table 14 Recovery of organisms from a defined inoculum post incubation in CDFF, utilising a number of selective and non-selective media. Results reported in Mean $Log_{10.}$(n=5)

	Day 1	**Day 3**	**Day 5**	**Day 8**
Total Count	9.6±0.8	10.7±2.3	10.5±1.2	10.4±1.8
Gram-ve	5.7±1.4	5.7±2.1	6.5±1.3	6.5±1.3
***Streptococcus* spp.**	7.5±1.2	8.5±1.1	8.7±2.0	8.6±0.7
***Lactobacillus* spp.**	5.5±0.9	5.6±0.9	6.5±1.5	6.5±0.5
S. mutans	6.6±0.8	5.7±1.2	7.4±0.9	7.6±1.3
***Neisseria* spp.**	6.5±1.3	7.3±0.3	7.6±1.7	7.6±0.9
***Actinomyces* spp.**	4.7±2.1	5.2±0.5	5.8±0.6	6.1±0.7
***Fusobacterium* spp.**	4.9±1.1	5.1±1.0	5.6±1.4	5.6±1.6
***Veillonella* spp.**	4.3±0.6	5.0±1.0	5.6±0.9	5.6±1.4

The results do show a decrease in *S. mutans* recovery at day 3. From the results received, subsequent biofilm analysis will be carried out on biofilms after 5 days incubation due to the relative stability seen with the bacterial recovery as seen in Figure 51.

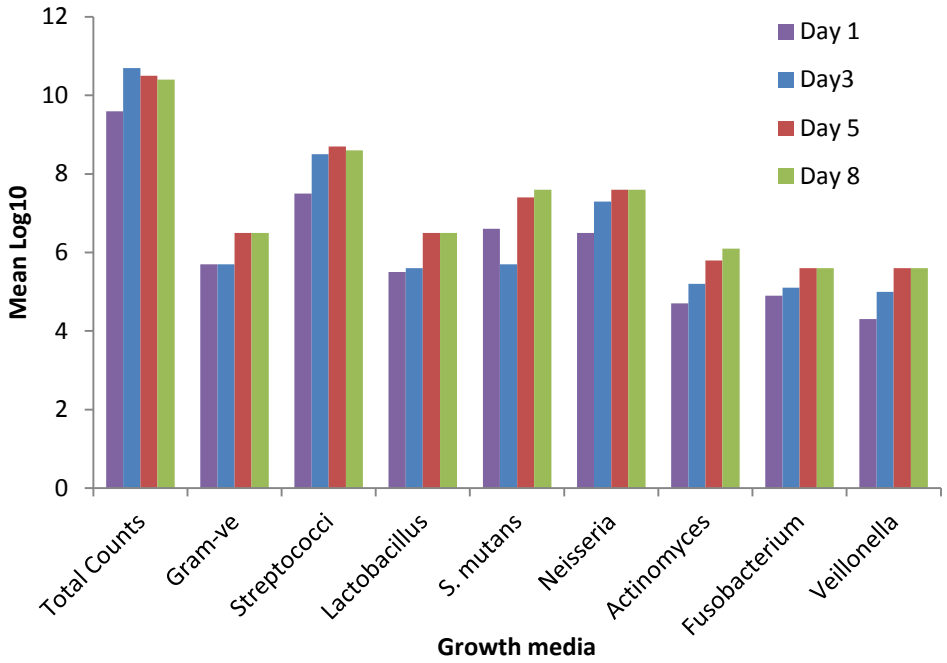


Figure 51 Graphical representation of bacterial recovery using a defined inoculum, post incubation at 1, 3, 5 and 8 days. Recovery on a number of selective and non-selective media, results reported in mean Log_{10}.

From the results in Figure 51, it is seen a steady increase in bacterial counts, after 8 days a number of the results show a 'settling' in bacterial counts, for example *Fusobacterium* sp., *Veillonella* sp. and *Neisseria* sp. If we look at the total counts, a slight decrease was found. In this study, it would, with hindsight, been beneficial to have extended the testing period to 15 days to understand more fully, whether these organisms were reaching steady state. However, it was decided that after 5 days incubation, it would be appropriate for the generation of suitable biofilms for further testing.

5.3.2 Antimicrobial Testing of Biofilms from the CDFF

5.3.2.1 Chlorhexidine Analysis

Bacterial recovery from the initial biofilms prior to exposure show reduced oral bacteria recovery than was seen previously in section 5.3.1. The results show that 0.5 % v/v CHX was sufficient to kill organisms in the biofilm, however at 0.1% and below, there was still recovery of some of the test organisms from the defined inoculum.

Table 15 Recovery of organisms from a defined inoculum post incubation in CDFF, utilising a number of selective and non-selective media. Biofilms were exposed to 0.5, 0.1 or 0.01% CHX. Results reported in Mean Log_{10}.

	Initial	**0.5% CHX**	**0.1% CHX**	**0.01% CHX**
Total Count	8.6±2.1	<1	5.4±0.7	7.4±2.0
Gram-ve	5.3±0.9	<1	4.3±0.6	5.1±1.5
***Streptococcus* spp.**	6.4±1.3	<1	4.7±0.4	5.2±0.5
***Lactobacillus* spp**	4.6±1.6	<1	4.1±0.6	4.6±0.8
S. mutans	4.9±1.6	<1	3.9±1.1	4.3±0.9
***Neisseria* spp.**	4.8±1.1	<1	3.7±0.9	4.3±0.3
***Actinomyces* spp.**	4.7±0.3	<1	<1	1.1±0.1
***Fusobacterium* spp.**	4.5±0.7	<1	<1	4.5±0.7
***Veillonella* spp**	4.5±1.6	<1	<1	3.2±1.1

Figure 52, shows graphically the reduction in bacterial viability with the increasing concentrations of CHX, where 0.5% CHX was sufficient to kill all organisms in the developed biofilms. With 0.01 and 0.1% CHX showing an overall reduction in bacterial numbers, but a retention of bacterial viability.

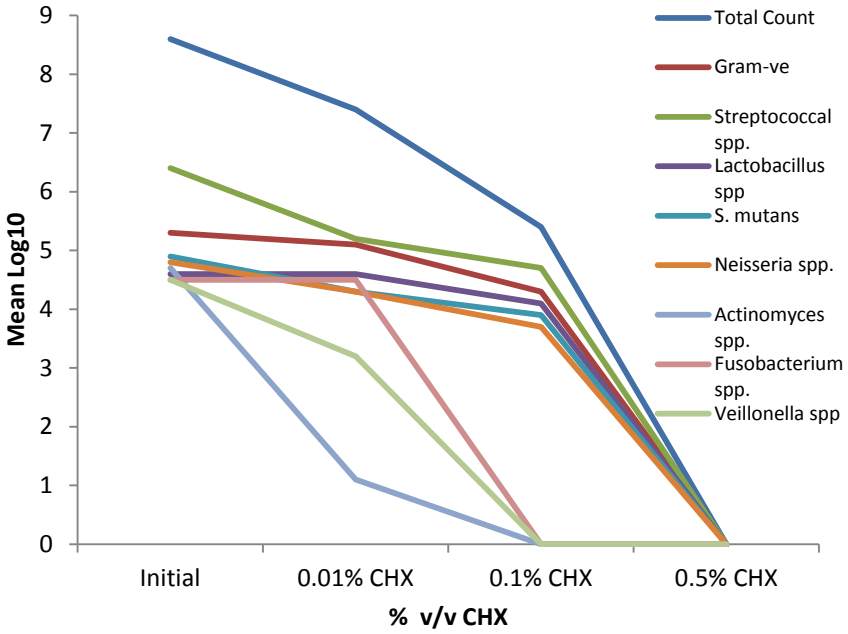


Figure 52 Graphical representation of bacterial recovery using a defined inoculum, post incubation of the CDFF for 5 days after exposure to 0.5, 0.1 or 0.01% v/v CHX. Recovery on a number of selective and non-selective media, results reported in mean Log_{10}.

5.3.2.2 Triclosan Analysis

The results below in Figure 53 show a reduction in antimicrobial activity in comparison to what was seen with the CHX in Figure 52, even at the highest concentrations tested. At the higher concentration of 1% w/v triclosan, an approximate 4 Log_{10} reduction in bacterial recovery was seen. There was a reduction in bacterial recovery of *Veillonella* spp. with 0.5% triclosan. It should be noted that there was a greater than 1 Log_{10} increase in total bacterial counts with the biofilms exposed to 0.5% w/v triclosan. There is no experimental hypothesis to support triclosan increasing bacterial viability at sub-lethal concentrations, rather the hypothesis that this may be excess biomass from the CDFF.

Table 16 Recovery of organisms from a defined inoculum post incubation in CDFF, utilising a number of selective and non-selective media. Biofilms were exposed to 1, 0.5 or 0.1% w/v triclosan. Results reported in Mean Log_{10} (n=5).

	Initial	1% Triclosan	0.5% Triclosan	0.1% Triclosan
Total Count	8.6±1.2	4.7±0.3	9.9±1.6	8.5±1.5
Gram-ve	5.3±0.9	4.4±0.6	4.8±1.7	5.2±0.6
***Streptococcus* spp.**	6.4±0.5	4.5±0.4	5.6±1.7	6.4±0.4
***Lactobacillus* spp**	4.6±1.1	<1	4.3±0.4	5.1±0.3
S. mutans	4.9±0.3	<1	4.4±0.5	4.8±1.2
***Neisseria* spp.**	4.8±0.7	<1	4.7±0.9	4.8±1.1
***Actinomyces* spp.**	4.7±0.8	<1	4.6±1.2	4.6±0.6
***Fusobacterium* spp.**	4.5±1.3	<1	4.5±0.3	4.5±0.5
***Veillonella* spp**	4.5±1.2	<1	1.9±0.5	4.6±0.8

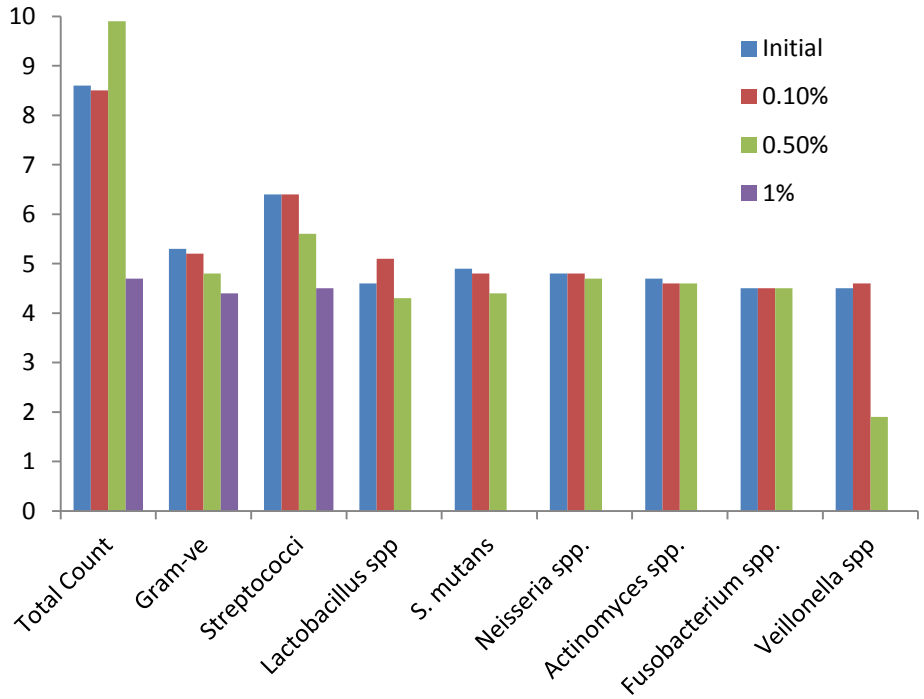


Figure 53 Graphical representation of bacterial recovery using a defined inoculum, post incubation of the CDFF for 5 days after exposure to 1, 0.5 or 0.1% w/v triclosan. Recovery on a number of selective and non-selective media, results reported in mean Log_{10}.

5.3.3 Minimum Biofilm Eradication Concentration Model

The results obtained from this study included all the individual organisms tested against each of the test agents, as well as the defined mix species inocula described in previous chapters.

5.3.3.1 Chlorhexidine Results

Using clorhexidine the effect was investigated in the developed biofilms using the MBEC biofilm model system.

5.3.3.1.1 Individual Species Biofilm Results using MBEC

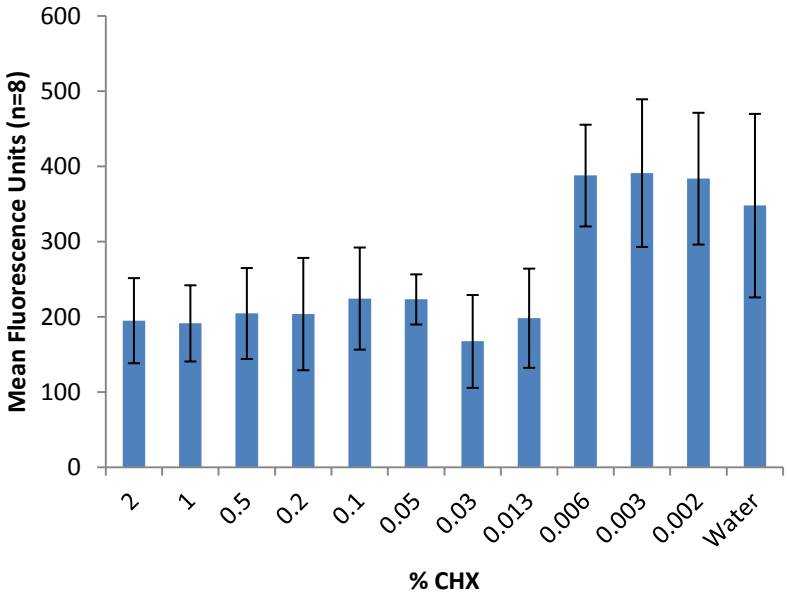


Figure 54 Effect of CHX (v/v) dose response on *S. mutans* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.006% v/v CHX concentrations.

The effect of CHX on *S. mutans* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	663435.0	11	60312.2	44.16	0.0000
Within groups	114712.0	84	1365.62		
Total (Corr.)	778147.0	95			

Treatments with the same letter are not significant

Method: 95.0 percent LSD

Treatment	Count	Mean	Homogeneous Groups
1	8	135.625	A
7	8	145.0	AB
2	8	147.5	AB
4	8	163.625	AB
5	8	166.125	AB
6	8	171.75	AB
3	8	177.125	B
8	8	254.625	C
9	8	263.75	C
11	8	338.375	D
10	8	353.0	D
12	8	361.375	D

Statistical analysis on the MBEC showed good seperation of the CHX dose response. It was found that treatment groups 1-7 (0.03 – 2% v/v CHX) did not split, where 2% w/w CHX was the positive control in this analysis. Treatment groups 8, 9 (0.006 – 0.013% w/w CHX) were similar, and treatment groups 10-12 (<0.003 w/w CHX) did not split from the water control (treatment 12).

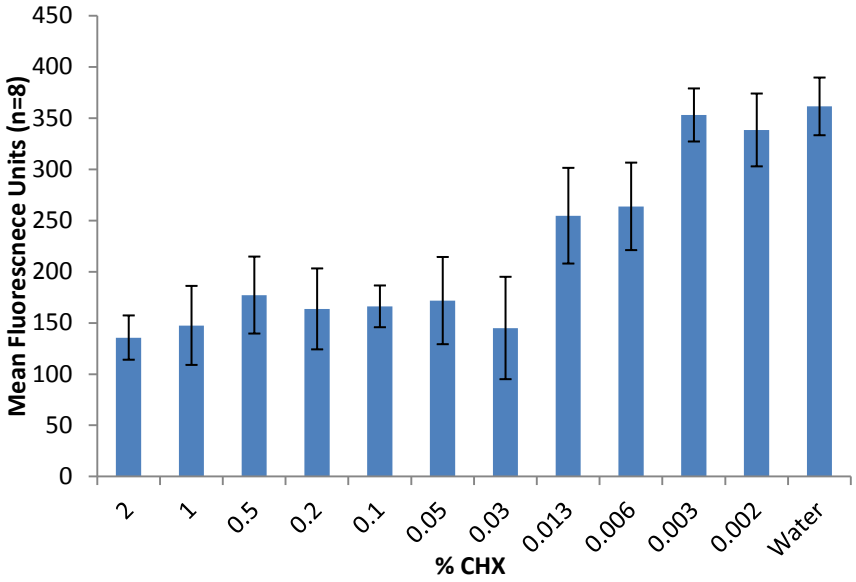


Figure 55 Effect of CHX (% v/v) dose response on *F. nucleatum* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.013% v/v CHX concentrations.

The effect of CHX on *F. nucleatum* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

Treatments with the same letter are not significant. There treatments 7-12 are statistically different to treatment 1-6, where treatment 1 is the 2% w/w CHX control and treatment 12 is the deionised water control.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatments    Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
2               8         174.5             A
1               8         222.125           AB
5               8         247.625            B
4               8         252.375            B
6               8         256.375            B
3               8         280.0              B
12              8         356.375              C
7               8         372.875              C
11              8         380.875              C
8               8         396.75               C
10              8         397.625              C
9               8         402.0                C
```

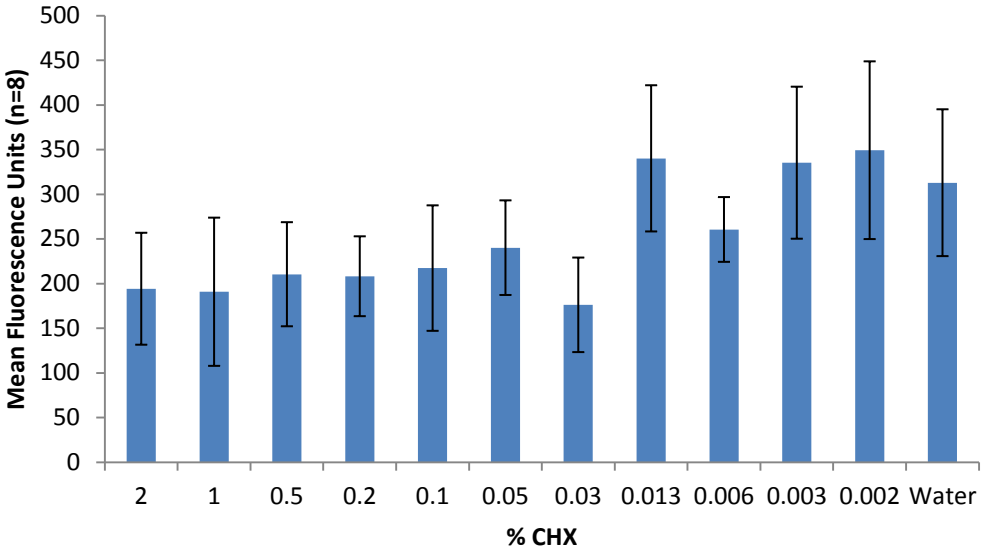


Figure 56 Effect of CHX (% v/v) dose response on *S. sanguinis* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.013% v/v CHX concentrations.

The effect of CHX on *S. sanguinis* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

Treatments with the same letter are not significant. The results show less differentiation between each of the test treatments, however treatments 8, 10-12 are statistically similar, where treatment 9 is not distinct from the water control (treatment 12). All other treatment regimes are not statistically different from the positive control 0.2% w/w CHX (treatment 1).

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Col_1          Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
7              8         176.125           A
2              8         190.75            A
1              8         194.125           AB
4              8         208.125           AB
3              8         210.375           AB
5              8         217.25            AB
6              8         240.125           AB
9              8         260.5              BC
12             8         312.875              CD
10             8         335.25                D
8              8         340.125               D
11             8         349.25                D
```

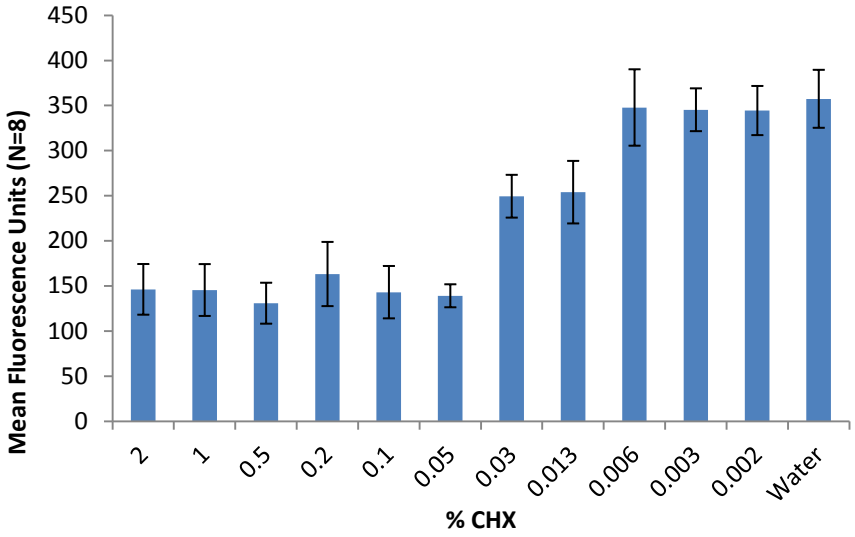


Figure 57 Effect of CHX (% v/v) dose response on *S. mitis* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.03% v/v CHX concentrations.

The effect of CHX on *S. mitis* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

Treatments with the same letter are not significant. Treatments 9-12 were statatistically similar to the negative control deionised water, treatments 1-6 were statistically similar to the positive control 2% w/w CHX. Treatments 7 and 8, although similar to each other, were not statistically similar to either the negative or positive controls.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatments   Count      Mean              Homogeneous Groups
------------------------------------------------------------------------------
3               8         130.75            A
6               8         139.0             AB
5               8         143.0             AB
2               8         145.375           AB
1               8         146.125           AB
4               8         163.125            B
7               8         249.375              C
8               8         253.875              C
11              8         344.375                D
10              8         345.25                 D
9               8         347.75                 D
12              8         357.375                D
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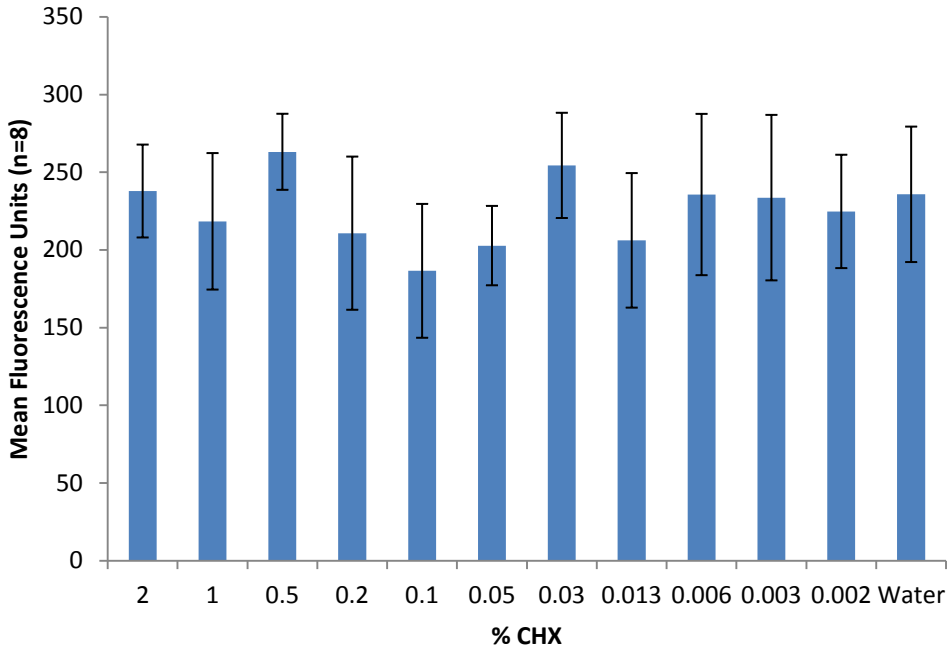

Figure 58 Effect of CHX (% v/v) dose response on *A. viscosus* biofilm viability using the MBEC model system (n=8). Where all treatments were statistically similar, where p=0.0153.

The effect of CHX on *A. viscosus* viability was analysed using a one-way analysis of variance with a factor for treatment, where p=0.0153. All the treatments, against *A. viscosus*, were statistically similar.

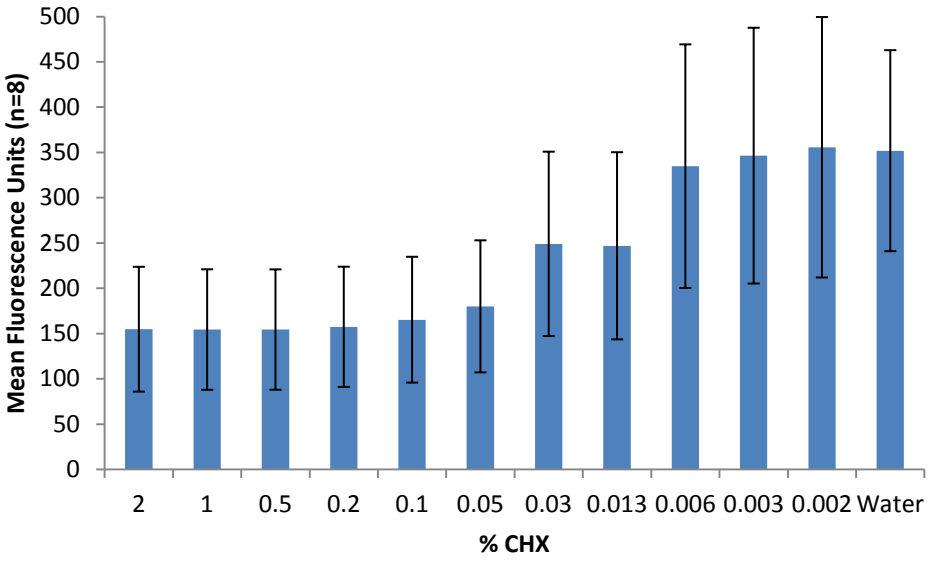

Figure 59 Effect of CHX (% v/v) dose response on *L. casei* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher that 0.03% CHX concentrations.

The effect of CHX on *L. casei* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$. Treatments with the same letter are not significant. Treatments 10-12 were statatistically similar to the negative control deionised water, treatments 1-6 were statistically similar to the positive control 2% w/w CHX. Treatments 7 and 8, although similar to each other, were not statistically similar to either the negative or positive controls.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment     Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
2               8         154.375          A
1               8         154.75           A
4               8         157.375          A
5               8         165.25           A
3               8         173.25           A
6               8         180.0            A
8               8         246.875           B
7               8         249.0             B
9               8         334.75              C
10              8         346.375             C
12              8         351.875             C
11              8         355.625             C
```

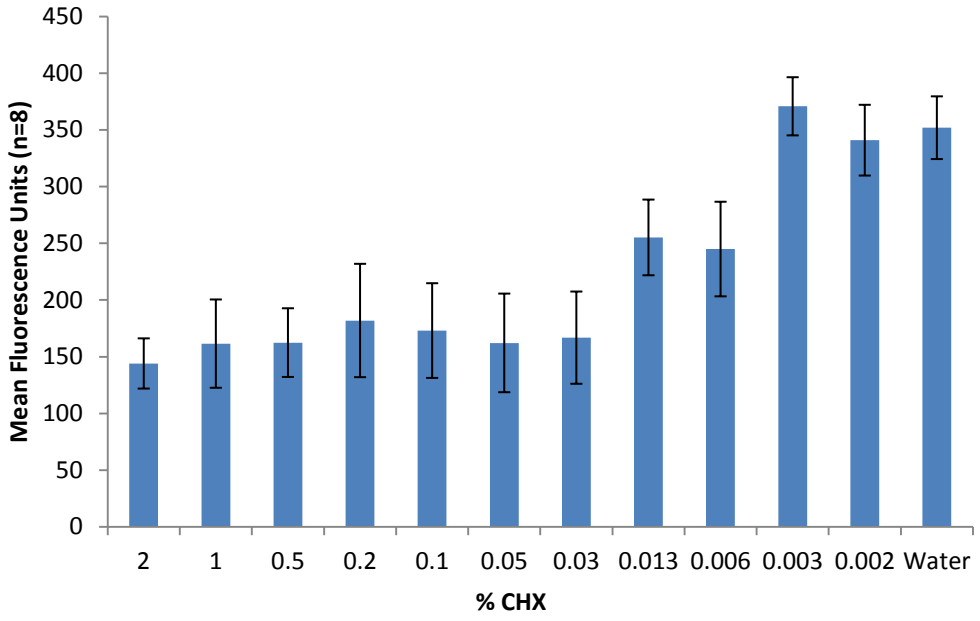

Figure 60 Effect of CHX (% v/v) dose response on *N. mucosa* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.03% v/v CHX concentrations

The effect of CHX on *N. mucosa* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$. Treatments with the same letter are not significant. Treatments 10-12 were statatistically similar to the negative control deionised water, treatments 1-7 were statistically similar to the positive control 2% w/w CHX. Treatments 8 and 9, although similar to each other, were not statistically similar to either the negative or positive controls.

Method: 95.0 percent LSD

Treatment	Count	LS Mean	LS Sigma	Homogeneous Groups
1	8	144.0	12.8914	A
2	8	161.5	12.8914	AB
6	8	162.125	12.8914	AB
3	8	162.375	12.8914	AB
7	8	166.75	12.8914	AB
5	8	173.0	12.8914	AB
4	8	181.875	12.8914	B
9	8	244.875	12.8914	C
8	8	255.125	12.8914	C
11	8	340.875	12.8914	D
12	8	351.875	12.8914	D
10	8	370.75	12.8914	D

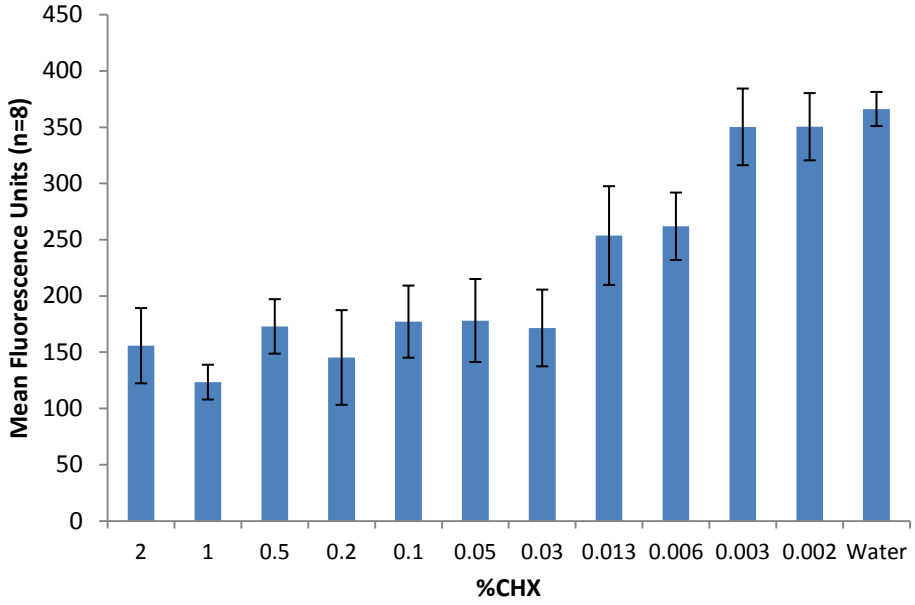

Figure 61 Effect of CHX (% v/v) dose response on *V. dispar* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.03% v/v CHX concentrations

The effect of CHX on *V. dispar* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$. Treatments with the same letter are not significant. Treatments 10-12 were statatistically similar to the negative control deionised water, treatments 1-7 were statistically similar to the positive control 2% w/w CHX. Treatments 8 and 9, although similar to each other, were not statistically similar to either the negative or positive controls

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment      Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
2              8         123.375            A
4              8         145.25             AB
1              8         155.75              BC
7              8         171.5               BC
3              8         172.875             BC
5              8         177.125             BC
6              8         178.125              C
8              8         253.625                D
9              8         261.875                D
10             8         350.25                   E
11             8         350.375                  E
12             8         366.125                  E
```

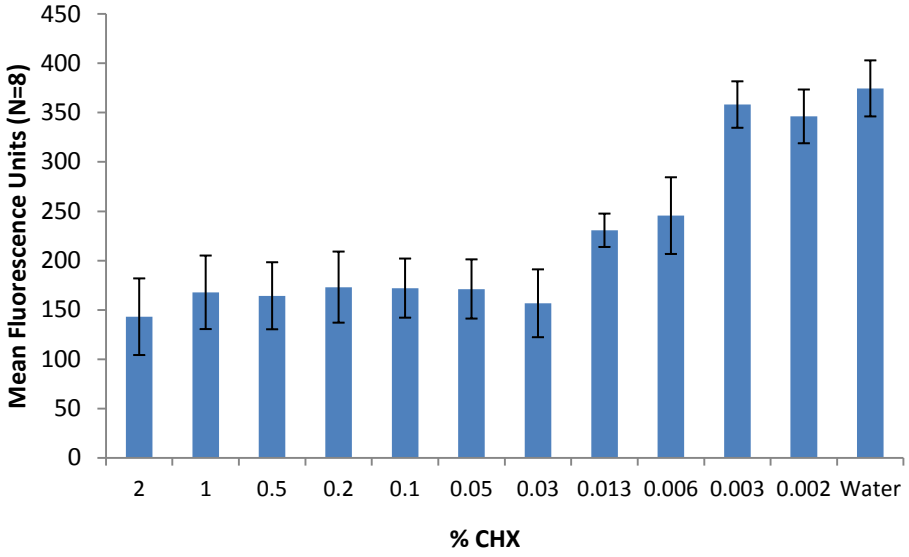

Figure 62 Effect of CHX (% v/v) dose response on *P. melaninogenicus* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.03% v/v CHX concentrations.

The effect of CHX on *P. melaninogenicus* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$. Treatments with the same letter are not significant. Treatments 10-12 were statatistically similar to the negative control deionised water, treatments 1-7 were statistically similar to the positive control 2% w/w CHX. Treatments 8 and 9, although similar to each other, were not statistically similar to either the negative or positive controls.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Col_1          Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
1              8         143.0             A
7              8         156.625           A
3              8         164.25            A
2              8         167.75            A
6              8         171.125           A
5              8         172.0             A
4              8         173.0             A
8              8         230.625            B
9              8         245.5              B
11             8         346.125              C
10             8         358.125              C
12             8         374.5                C
```

The results show, using the individual organisms, attached organisms to the pegs can be evaluated for their sensitivity to antimicrobial agents. It should be noted that it was anticipated that the MFU would be higher. In these studies reported here, the MFU is very low using this methodology, this was seen across all the analyses carried out. This may suggest that there may not be many organisms attached to the pegs. Indeed, this is supported by the confocal results later in this chapter

5.3.3.1.2 Mixed Species Defined Biofilm Results using MBEC

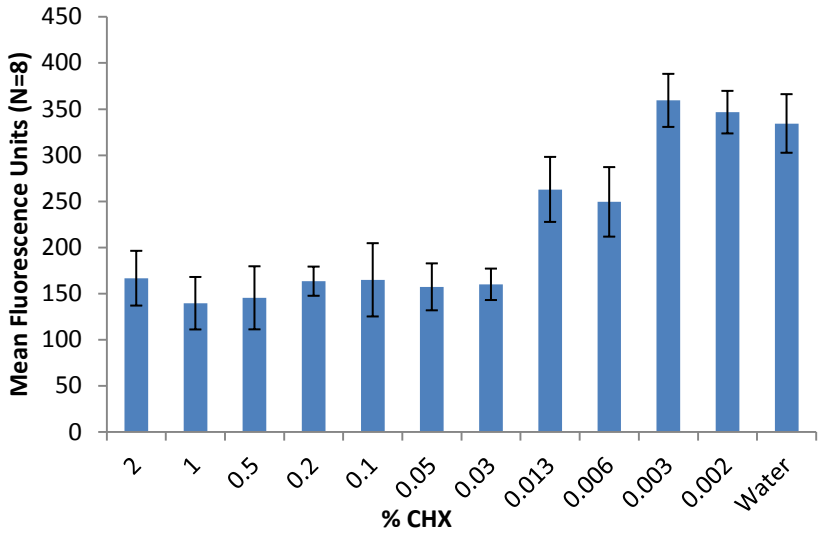

Figure 63 Effect of CHX (% v/v) dose response to a mixed species biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at 0.03% v/v CHX and higher.

The effect of CHX on mixed biofilm viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$. Treatments with the same letter are not significant. Treatments 10-12 were statatistically similar to the negative control deionised water, treatments 1-7 were statistically similar to the positive control 2% w/w CHX. Treatments 8 and 9, although similar to each other, were not statistically similar to either the negative or positive controls

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment          Count     LS Mean       LS Sigma      Homogeneous Groups
------------------------------------------------------------------------------
2                  8         139.5         10.5344       A
3                  8         145.375       10.5344       A
6                  8         157.25        10.5344       A
7                  8         160.0         10.5344       A
4                  8         163.375       10.5344       A
5                  8         164.875       10.5344       A
1                  8         166.625       10.5344       A
9                  8         249.375       10.5344        B
8                  8         262.875       10.5344        B
12                 8         334.375       10.5344         C
11                 8         346.625       10.5344         C
10                 8         359.375       10.5344         C
```

5.3.3.2 Triclosan Results

The low MFU results were also found with the following study.

5.3.3.2.1 Individual Species Biofilm Results using MBEC

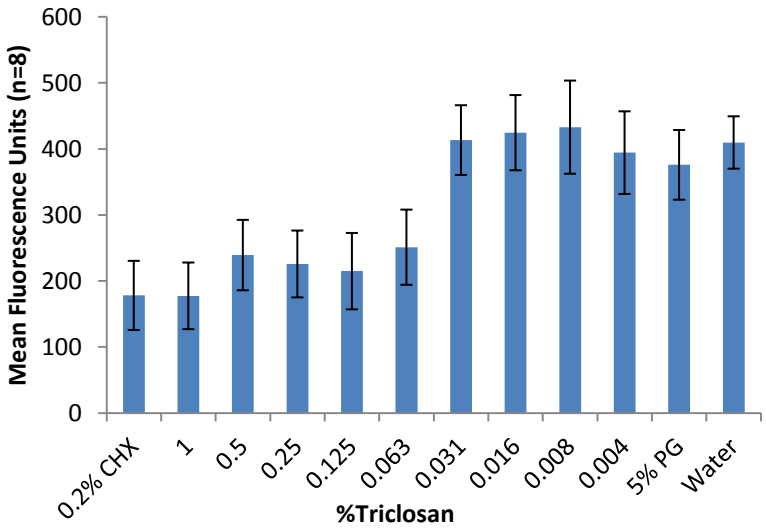

Figure 64 Effect of Triclosan (% w/v) dose response on *S. mutans* biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.063% w/v triclosan concentration.

The effect of triclosan on *S. mutans* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment     Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
2               8         177.5             A
1               8         178.125           A
5               8         214.875           AB
4               8         225.75            AB
3               8         239.25             B
6               8         251.125            B
11              8         375.875              C
10              8         394.375              CD
12              8         409.75               CD
7               8         413.375              CD
8               8         424.625              CD
9               8         433.0                  E
```

Treatments with the same letter are not significant. Treatments 7-12 were statatistically similar to the negative control deionised water, treatments 1-6 were statistically similar to the positive control 0.2% w/w CHX.

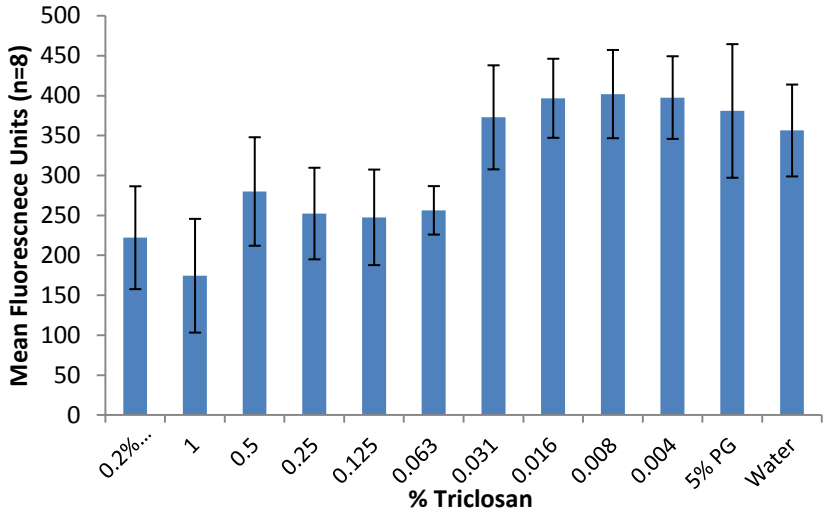

Figure 65 Effect of Triclosan (% w/v) dose response on *F. nucleatum* biofilm viability using the MBEC model system (n=8). The results showing a slight reduction in biofilm viability.

The effect of triclosan on *F. nucleatum* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$. Treatments with the same letter are not significant. Treatments 7-12 were statatistically similar to the negative control deionised water, treatments 1-6 were statistically similar to the positive control 0.2% w/w CHX.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment     Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
2               8         177.5             A
1               8         178.125           A
5               8         214.875           AB
4               8         225.75            AB
3               8         239.25             B
6               8         251.125            B
11              8         375.875              C
10              8         394.375              CD
12              8         409.75               CD
7               8         413.375              CD
8               8         424.625              CD
9               8         433.0                 D
```

5.3.3.2.2 Mixed Species Biofilm Results using MBEC

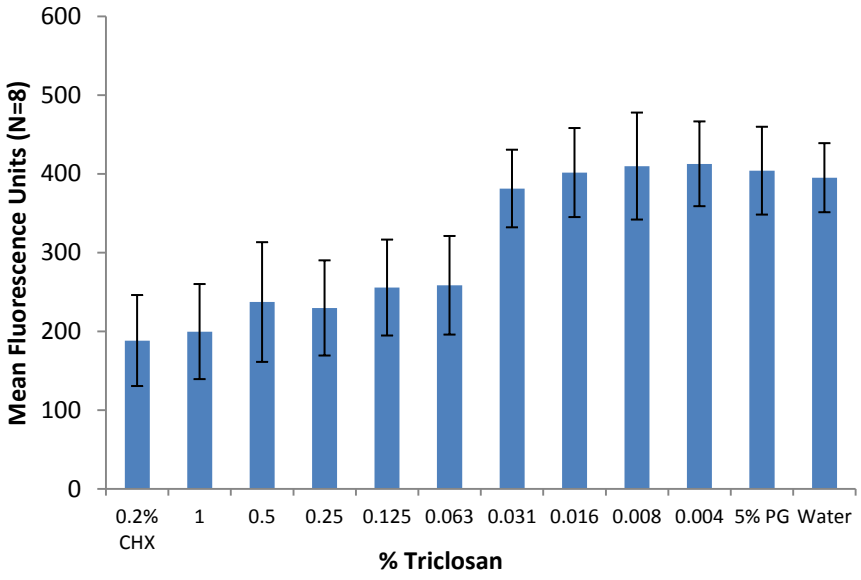

Figure 66 Effect of Triclosan (% w/v) dose response on a defined inoculum biofilm viability using the MBEC model system (n=8). Showing a reduction in biofilm viability at higher than 0.063% w/v triclosan concentration.

The effect of triclosan on biofilm viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment     Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
1               8         188.375           A
2               8         199.75            AB
4               8         229.75            AB
3               8         237.25            AB
5               8         255.625            B
6               8         258.5              B
7               8         381.375              C
12              8         395.125              C
8               8         401.625              C
11              8         404.0                C
9               8         409.875              C
10              8         412.75               C
```

Treatments with the same letter are not significant. Treatments 7-12 were statatistically similar to the negative control deionised water, treatments 1-6 were statistically similar to the positive control 0.2% w/w CHX.

All results received show similar dose response pattern to that seen with the CHX, however, in this study, triclosan was less effective at lower concentrations, less than 0.063% w/v triclosan, where as CHX was found to be effective below 0.03% v/v CHX. Again, the results show a reduced MFC than would be anticipated.

5.3.3.3 Zinc Chloride Results

The results below show the effect of the zinc chloride against biofilm viability ising the MBEC model. The low MFU results can be seen with the following study results also.

5.3.3.3.1 Individual Species Biofilm Results using MBEC

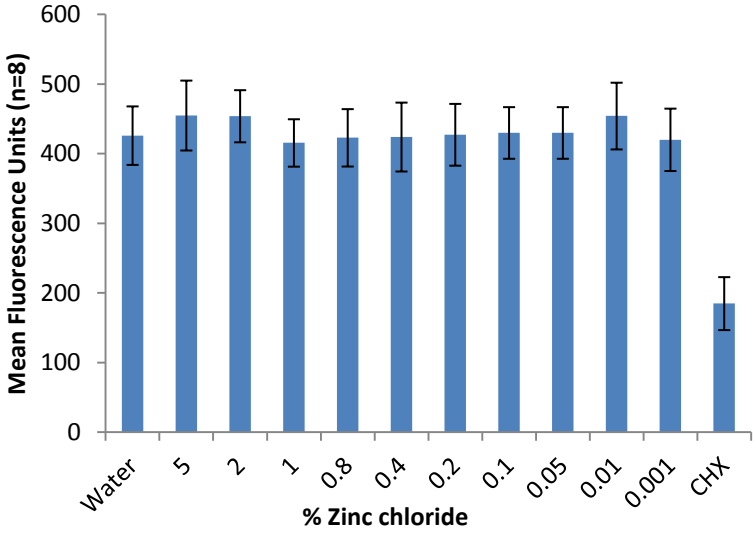

Figure 67 Effect of Zinc chloride (% w/v) dose response on *S. mutans* biofilm viability using the MBEC model system (n=8). Showing a no reduction in biofilm viability at any concentration tested.

The effect of zinc chloride on *S. mutans* viability was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment     Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
12              8         184.75             A
4               8         415.5                B
11              8         420.0                B
5               8         422.875              B
6               8         424.0                B
1               8         426.0                B
7               8         427.25               B
9               8         429.875              B
8               8         429.875              B
3               8         453.875              B
10              8         454.125              B
2               8         454.875              B
```

Treatment with the same letter are not significant. Where treatment 1-11 are distinct from the positive control, treatment 12.

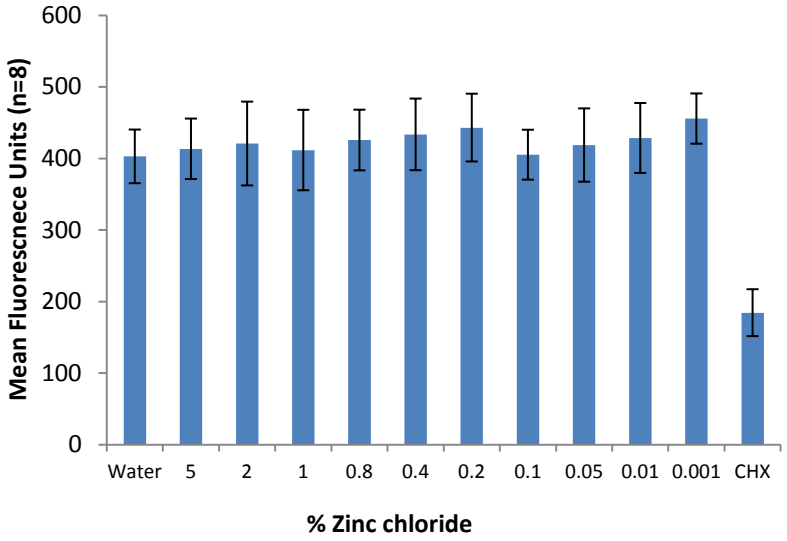

Figure 68 Effect of Zinc chloride (% w/v) dose response on *F. nucleatum* biofilm viability using the MBEC model system (n=8). Showing no reduction in biofilm viability at any concentration tested.

The effect of zinc chloride on F. nucleatum biofilm viability was analysed using a one-way analysis of variance with a factor for treatment. Treatments with the same letter are not significant, where treatment 1-11 were statistically similar to the water control, and statistically distinct from the positive control CHX.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment    Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
12             8         184.375           A
1              8         402.75             B
8              8         405.125            B
4              8         411.625            BC
2              8         413.375            BC
9              8         418.625            BC
3              8         420.75             BC
5              8         425.625            BC
10             8         428.5              BC
6              8         433.5              BC
7              8         443.0              BC
11             8         455.625             C
```

5.3.3.3.2 Mixed Species Biofilm Results using MBEC

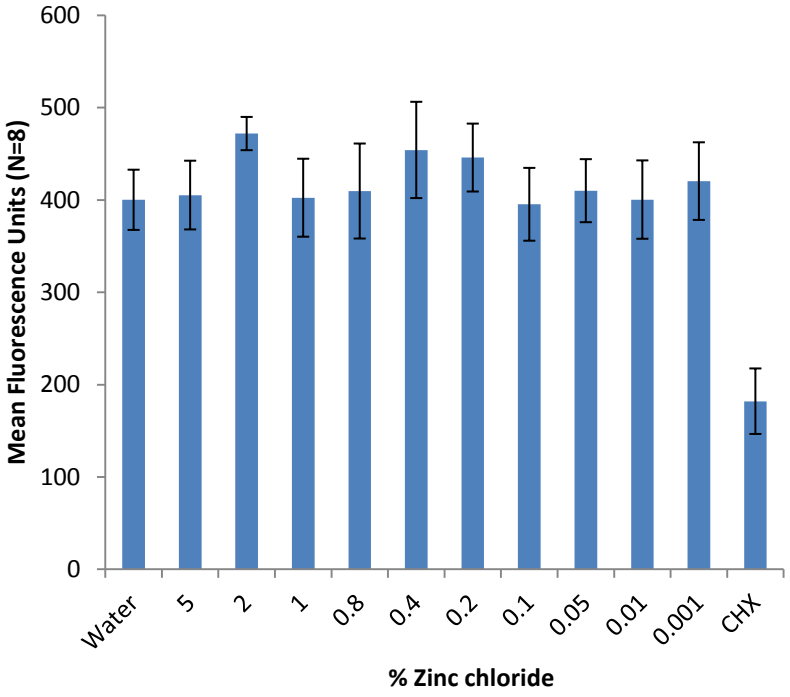

Figure 69 Effect of Zinc chloride (% w/v) dose response on a mixed species biofilm viability using the MBEC model system (n=8). Showing no reduction in biofilm viability across any tested concentration.

The effect of zinc chloride on mixed species biofilm viability was analysed using a one-way analysis of variance with a factor for treatment. Treatments with the same letter are not significant, where treatment 1-11 are statistically similar to the water control, and statistically distinct from the CHX control (treatment 12).

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Col_1          Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
12             8         182.0             A
8              8         395.25             B
1              8         400.125            B
10             8         400.375            B
4              8         402.375            B
2              8         405.25             B
5              8         409.625            BC
9              8         410.0              BC
11             8         420.375            BCD
7              8         445.875             CDE
6              8         454.125               DE
3              8         471.875                E
```

In this study, no reduction in attached bacteria viability was seen across all the concentrations tested for all organisms or the mixed species inoculums.

5.3.4 Developed Bioplate Model Results

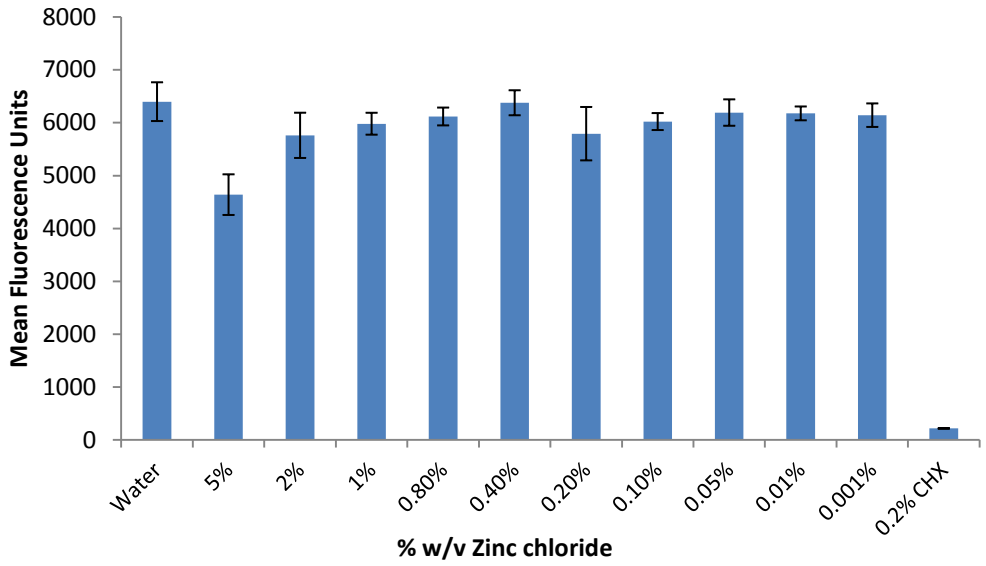

Figure 70 Zinc chloride dose response in the developed Bioplate, reduction in viability is seen with 5% w/v Zinc chloride, no reduction in comparison with water control is seen <5% w/v zinc chloride. (n=8).

The effect of zinc chloride on mixed species biofilm viability using the Bioplate was analysed using a one-way analysis of variance with a factor for treatment.

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Treatment     Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
12              8         218.0            A
2               8         4639.63           B
3               8         5761.25             C
7               8         5792.88             C
4               8         5980.88             CD
8               8         6022.13             CD
5               8         6117.88               DE
11              8         6142.63               DE
10              8         6176.5                DE
9               8         6192.25               DE
6               8         6377.63                E
1               8         6398.25                E
```

Treatments with the same letter are not significant, where treatment 12 (positive control 0.2% CHX was statistically different to all treatments. Five percent zinc chloride showed a reduction in bacterial viability, all other test treatments are not statistically distince from each other. The results from the Bioplate assay show a reduction in biofilm viability with the 5% w/v zinc chloride, however at all other concentrations there was no reduction in viability seen. In this assay water and 0.2% CHX were used as negative and positive controls respectively.

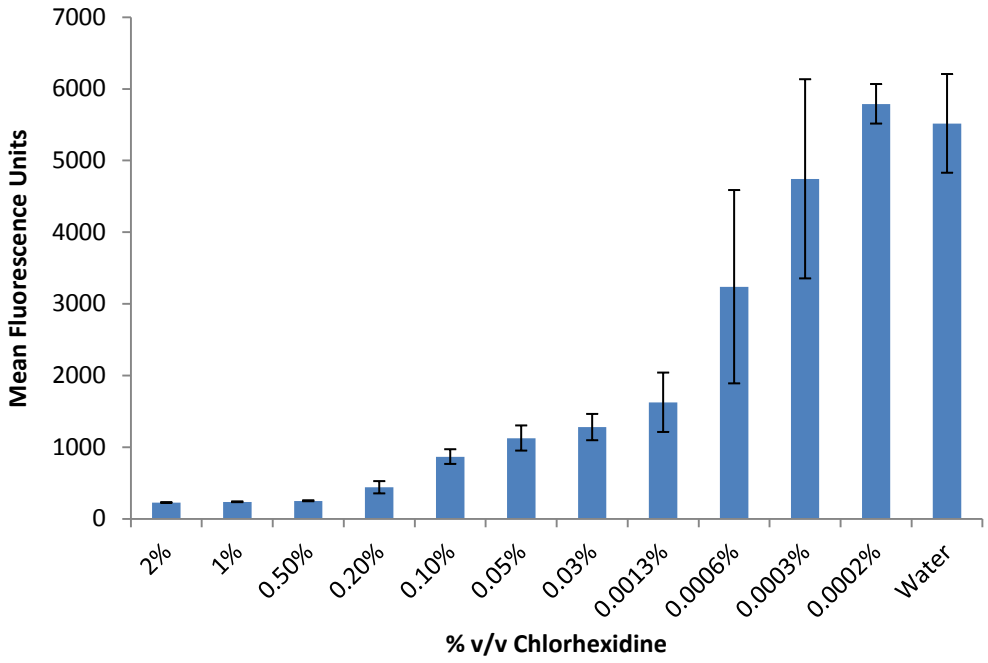

Figure 71 Chlorhexidine dose response in the developed Bioplate, reduction in viability is seen >0.006% CHX. A reduction in viability is seen until viability is similar to that of the water control (n=8).

The effect of CHX on mixed species biofilm viability using the Bioplate was analysed using a one-way analysis of variance with a factor for treatment. Treatments with the same letter are not significant, where treatment 1 (positive control 2% CHX was statistically similar to all concentrations tested to 0.2%

CHX. Treatments from 0.0013-0.1% CHX are not statistically dissimilar, treatments 9-11 show good statistical differentiation, and treatments 11 and 12 are statistically similar where treatment 12 is the negative water control in this model.

Method: 95.0 percent LSD			
Treatments	Count	Mean	Homogeneous Groups
1	8	225.25	A
2	8	236.0	A
3	8	250.125	A
4	8	438.75	AB
5	8	866.875	BC
6	8	1126.0	CD
7	8	1278.63	CD
8	8	1624.63	D
9	8	3237.75	E
10	8	4743.75	F
12	8	5517.38	G
11	8	5790.5	G

The results using the Bioplate assay against a CHX dose response show good reduction in biofilm viability which starts to drop significantly at 0.006% w/v CHX. Water is used as a negative control in this assay. Two per cent CHX (v/v) was utilised as both a positive control and test active in this study.

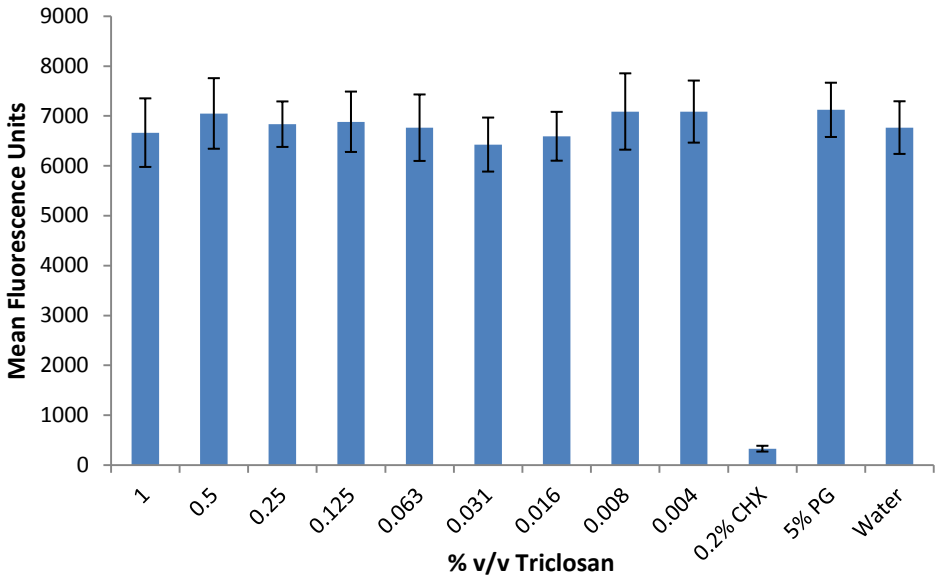

Figure 72 Triclosan dose response in the developed Bioplate, reduction in viability is seen not seen at any concentrations tested in comparison to the water control. (n=8).

The effect of triclosan on mixed species biofilm viability using the Bioplate was analysed using a one-way analysis of variance with a factor for treatment. Treatments with the same letter are not significant, where treatment 10 (positive control 2% CHX was statistically different to all triclosan concentrations evaluated

```
------------------------------------------------------------------------------
Method: 95.0 percent LSD
Col_1          Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
10             8         328.0             A
6              8         6425.25            B
7              8         6591.38            BC
1              8         6664.38            BC
5              8         6763.5             BC
12             8         6765.63            BC
3              8         6834.13            BC
4              8         6882.63            BC
2              8         7049.0              C
9              8         7086.25             C
8              8         7088.13             C
11             8         7121.5              C
```

There is no reduction in biofilm viability seen with triclosan at all concentrations tested post two minutes exposure. In this study 0.2% CHX was used as a positive control, 5% propylene glycol (PG) and water are used as negative controls, where 5% PG is the solubolising agent for triclosan.

Across the models evaluated, consistently the Bioplate gave MFU counts that gave clearer differentiation of results. To understand this more fully, samples were taken of the biofilms and evaluated using Live/Dead BacLight to understand the biomass formed on each of the surfaces.

In this evaluation it was very clear that Bioplate model set up and turn around was some what shorter than that of the MBEC and CDFF model systems. Where the inocula could be pre-prepared and stored, thus reducing this initial bacteria recovery, incubation and inocula generation everytime a Bioplate was required.

5.3.5 Sorbarod Biofilm Model System

Three Sorbarod biofilms were run in parallel and each acted as their own baseline control. The data presented are representative runs of three separate runs. Environmental scanning electron micrographs of an uninoculated Sorbarod (Figure 73) show individual cellulose fibres that act as the surface for attachment and colonization of tongue-derived bacteria.

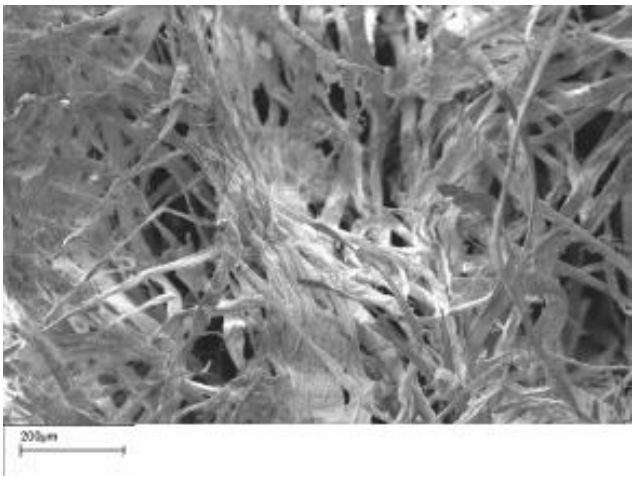

Figure 73 Sterile Sorbarod Filter, micrograph showing web of cellulose fibres in an uninoculated Sorbarod filter (bar = 200 µm)

Images of a 48 h tongue biofilm show significant material between and associated with the cellulose fibres (Figure 74). Individual bacteria or microcolonies were not evident; however the initial partial dehydration step in the environmental SEM may have collapsed the extracellular matrix material produced by most biofilms.

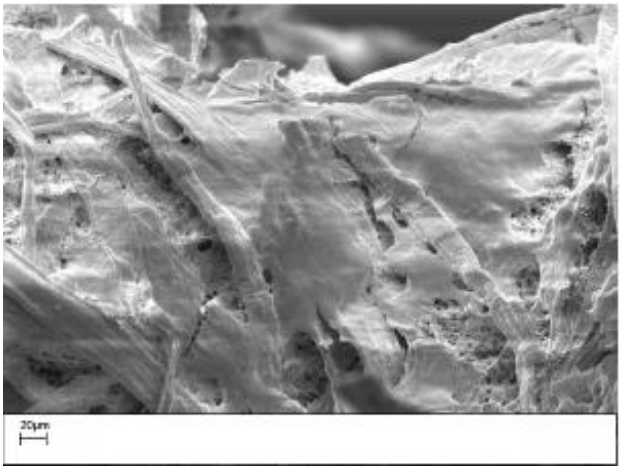

Figure 74 Inoculated Sorbarod Filter, micrograph of a 48 hour old inoculated Sorbarod showing smooth outer edge of biofilm with a number of large channels (bar = 20 µm)

5.3.5.1 Impact of Test Agents on Biofilm-Generated VSC Levels

The effects of various test actives on VSC production by the Sorbarod tongue biofilm model are shown below.

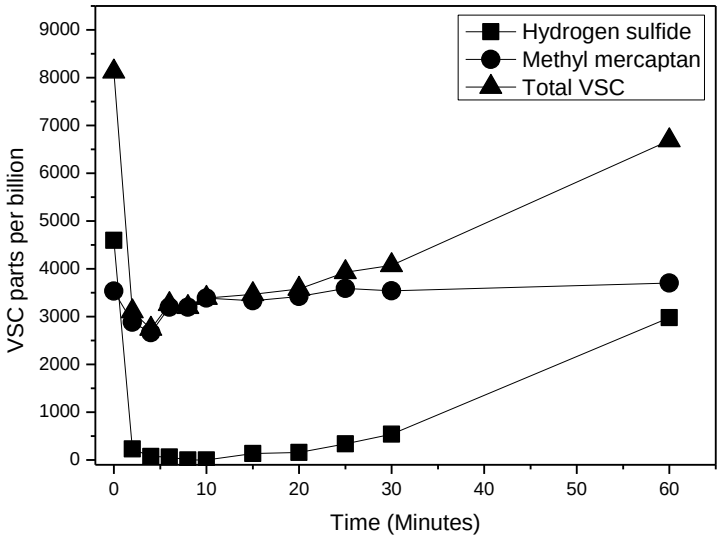

Figure 75 VSC concentrations after 1 minute exposure to 0.01% w/v zinc acetate. Forty eight hour-old inoculated Sorbarods were exposed to 0.1% w/v zinc acetate for 1 minutes. Gas samples were collected for up to 60 minutes post-exposure and analysed for VSC (ppb).

The metal salts tested (zinc acetate and copper (II) gluconate) had a large and immediate impact on hydrogen sulfide (H_2S) concentrations (Figure 75 and Figure 76) such that concentration were reduced to undetectable levels after 1 min exposure and remained at this concentration for approximately 20 min (zinc) or 30 min (copper). Even 60 min after exposure, H_2S concentration remained lower than at baseline.

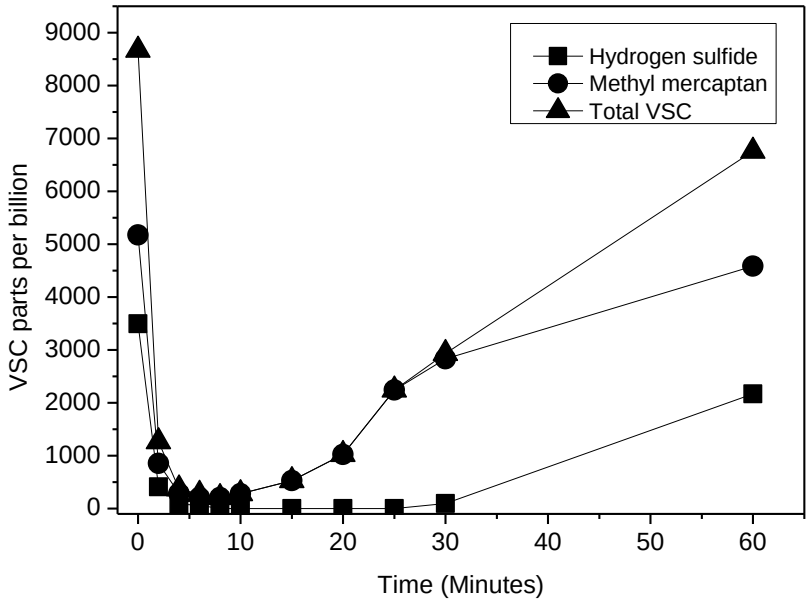

Figure 76 VSC concentrations after 1 minute exposure to 0.2% w/w copper (II) gluconate. 48 hour-old inoculated Sorbarods were exposed to 0.2% w/v copper (II) gluconate for 1 minute. Gas Samples were collected for up to 60 minutes post-exposure and analysed for VSCs (ppb).

In contrast, zinc and copper salts had differential effects on methyl mercaptan (CH_3SH) concentrations. While biofilm exposure to 0.1%w/v zinc acetate did not affect CH_3SH concentrations, 0.1%w/v copper (II) gluconate reduced CH_3SH concentrations to below detectable levels over 10 min. With both metal ions, H_2S and CH_3SH concentrations increased over time such that total VSC concentrations approached baseline after 60 min. Chlorhexidine is considered the gold-standard

antimicrobial active for oral care based, at least in part, due to its retention in an active form on oral surfaces (Kinniment *et al.*, 1996; McBain *et al.*, 2003; Pratten *et al*., 1998). Typical results from a 48 hour Sorbarod biofilm exposed to 0.2% v/v chlorhexidine are shown in Figure 77. An initial increase in total VSC concentrations of approximately 25% was observed followed by a return to baseline within 5 minutes.

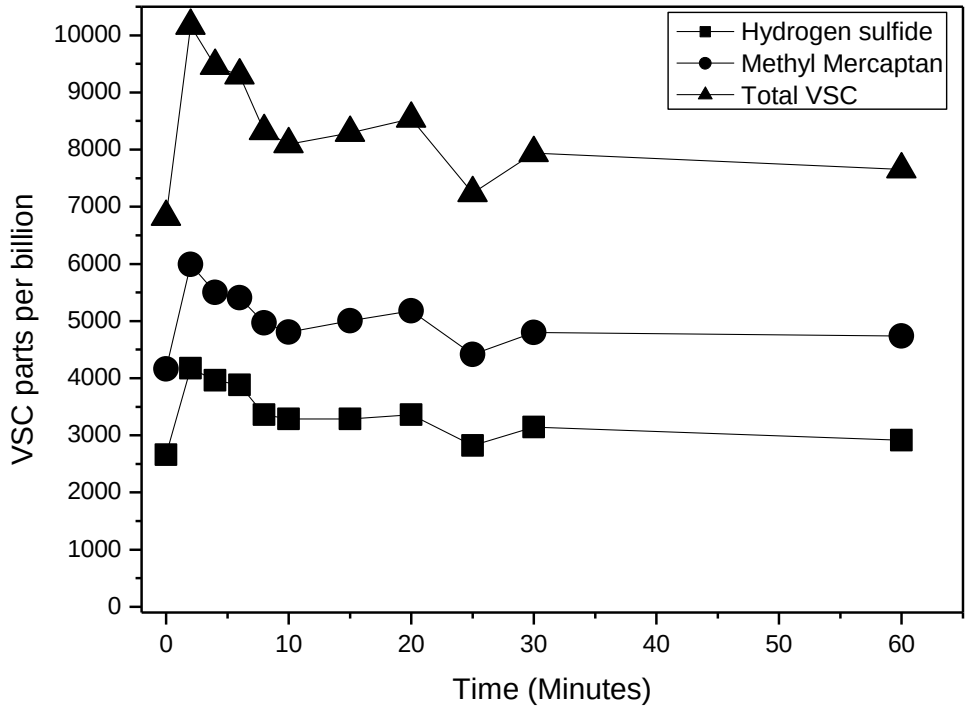

Figure 77. VSC concentrations after 48 hrs incubation using 0.2%v/v chlorhexidine 48 hour-old inoculated Sobarods were exposed to 0.2% v/v chlorhexidine for 1 minute. Gas samples were collected for up to 60 minutes post exposure and analysed for VSCs (ppb).

5.3.6 Confocal Laser Scanning Microscopy

The images below are for illustrative purposes only and no further analysis was carried out. The images are used to show the biomass which forms in comparison with the CDFF, MBEC and developed biofilm model.

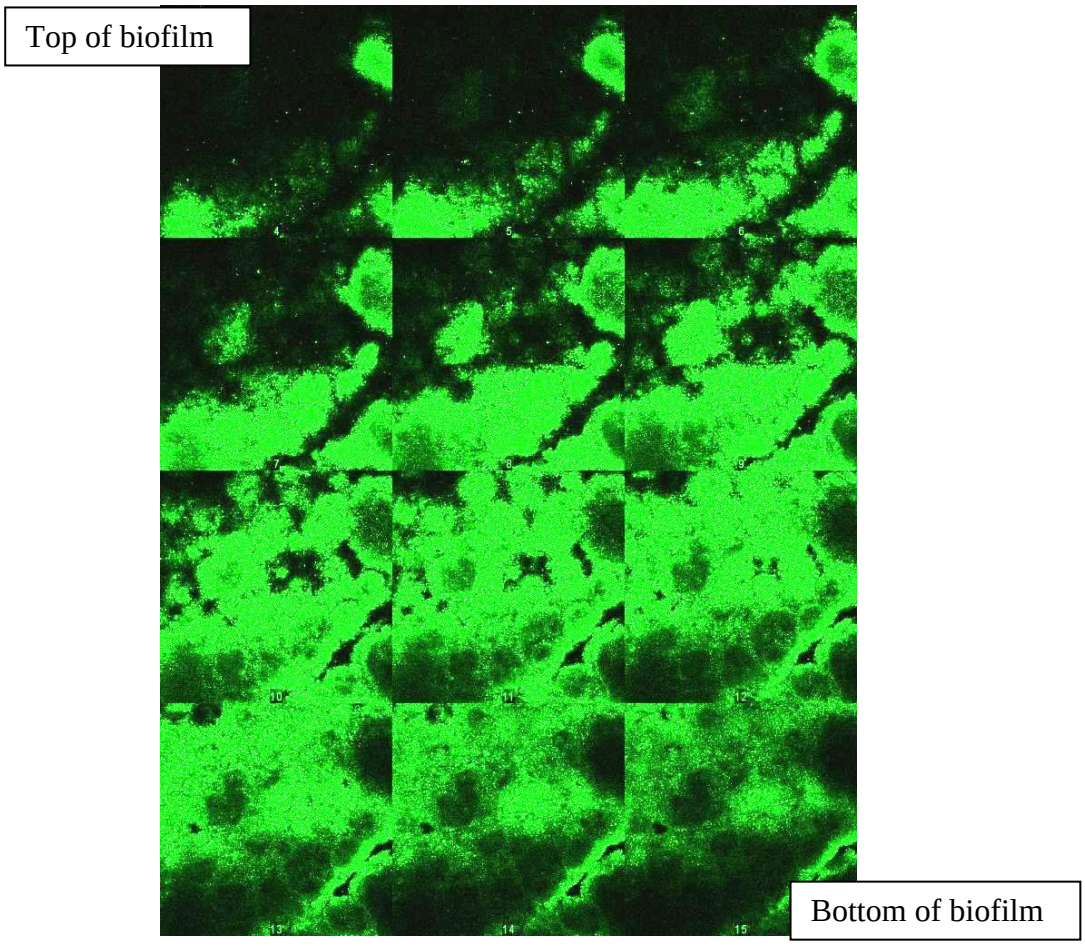

Figure 78 Optical sections (partial image stack) through a biofilm grown in a CDFF and stained with Live/Dead BacLight™ exhibiting structural motifs of dense matrix with channels. Each image 297.98x297.98 µm.

Using the CDFF to develop biofilms, reveal a dense bio-matrix full of channels. This type of image would be expected with the CDFF, due to the model, which scrapes biomaterial into the individual pans, resulting in dense biofilm formation.

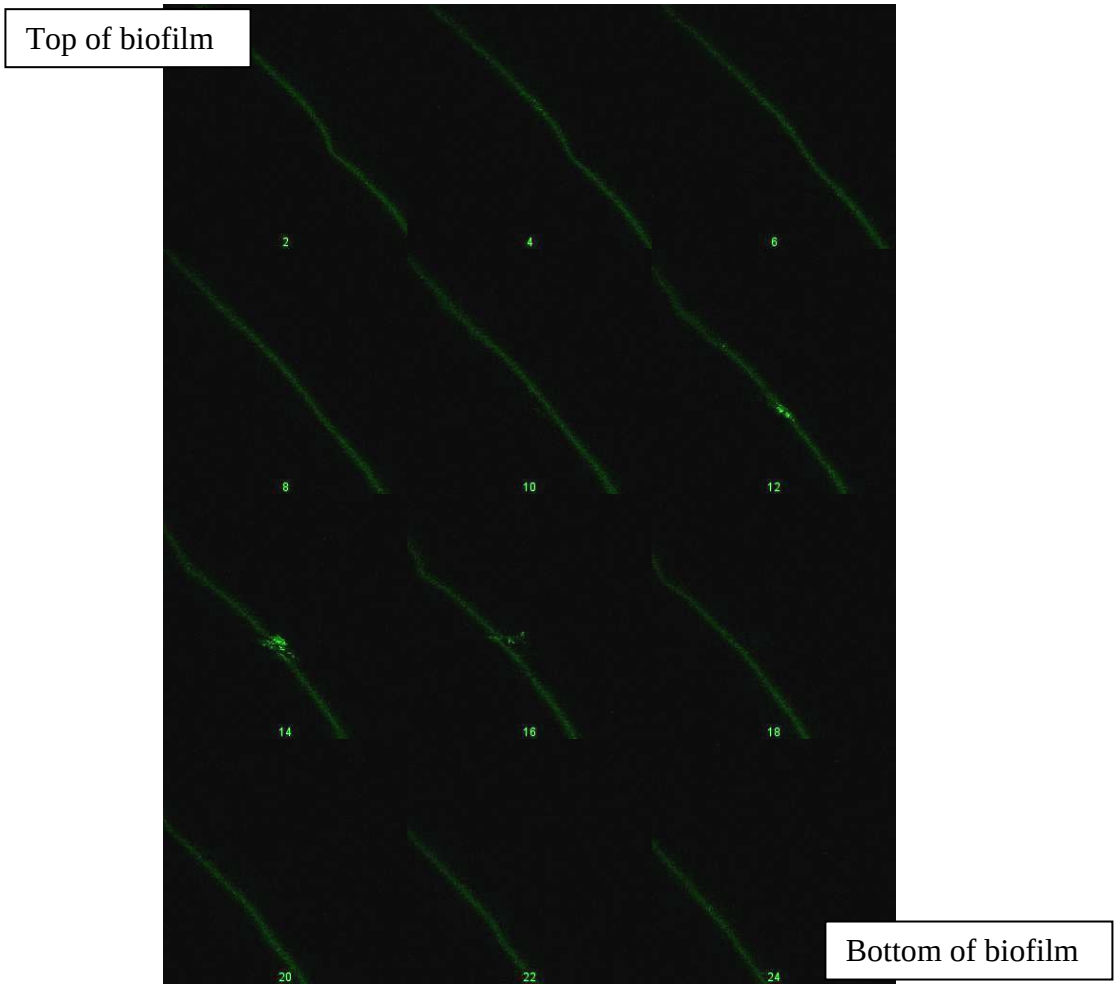

Figure 79 Optical sections (partial image stack) through a biofilm grown on an MBEC peg and stained with Live/Dead BacLight™ exhibiting little bacterial attachment and subsequent biofilm growth. Each image 297.89 x 297.98 µm.

In this micrograph, it was found that very little biofilm had developmed on the plastic pegs of the MBEC model, indeed, a small amount of attached organisms can be seen in image 12 and 14. The greem line which can bee seen in all images is reflection off the plastic pegs and should not be confused with viable organisms attached to the surface.

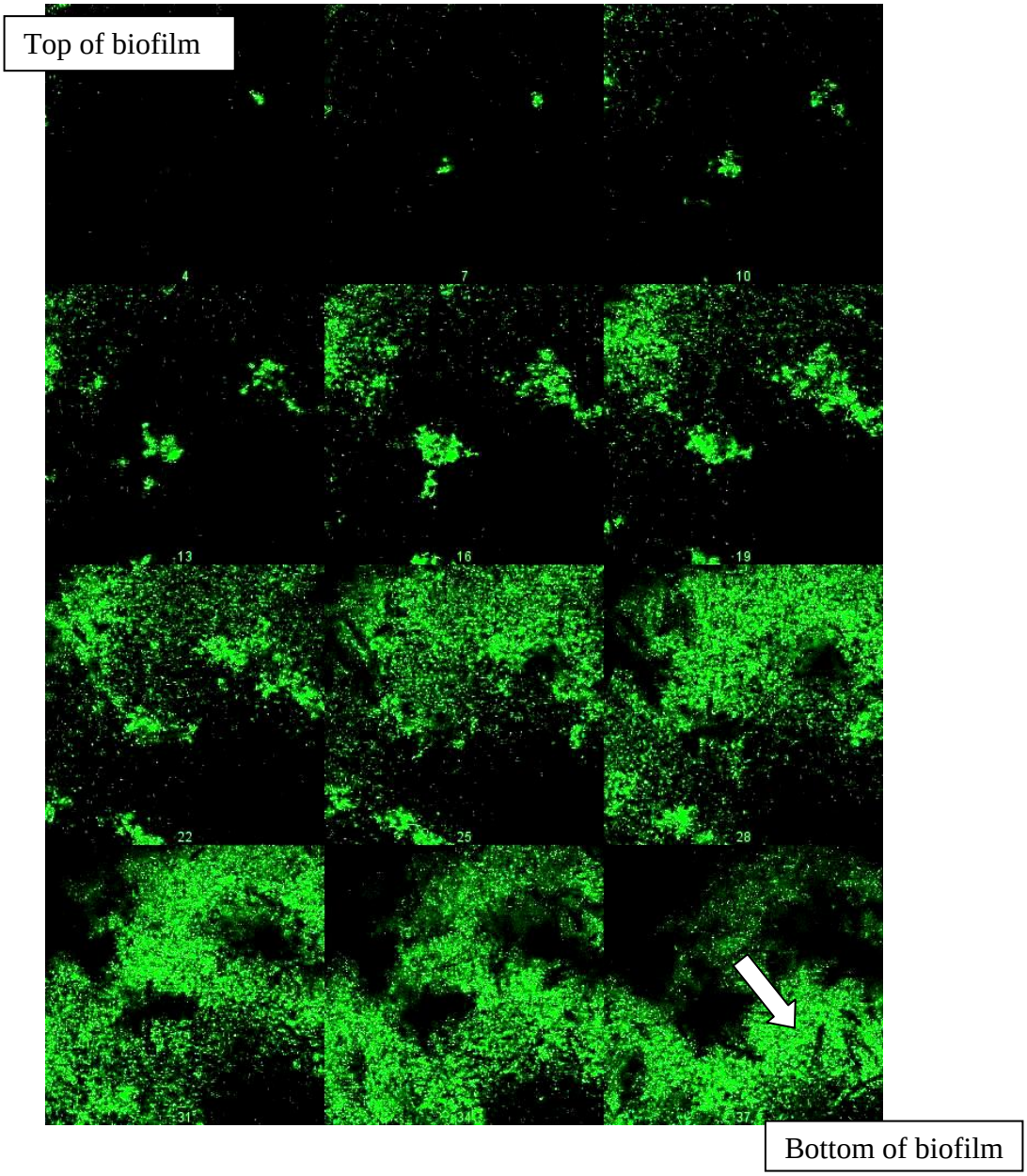

Figure 80 Optical sections (partial image stack) through a biofilm grown on the developed biofilm model system and stained with Live/Dead BacLight™ exhibiting structural motifs of dense matrix with channels. Arrow highlights what the author to believe to be HA crystals (dark void) with biomass forming around crystals. Each image 297.98 x 297.98 µm.

The developed biofilms from the Bioplate show structural motifs that are similar to those seen with the CDFF model, but slightly less dense, this is to be expeted as the biofilms are permitted to develop, undisturbed, where the CDFF model continually scrapes biomass into the recessed pans, compacting the developed biofilms.

5.4 Discussion

Currently, there are a number of peer reviewed biofilm model systems available for modelling biofilms found in the oral cavity, such as the CDFF, MBEC and Sorbarod. In this chapter, the developed HA-coated microtitre plate was evaluated against these peer reviewed model systems utilising a number of recognised oral care antimicrobial agents, chlorhexidine, triclosan and zinc chloride/acetate. A summary of the results can be found in Table 17.

Table 17 Summary of the results comparing each model system.

	Developed Bioplate	**CDFF**	**MBEC**	**Sorbarod**
Retention of mixed species biofilms	Yes	Yes	No	Not tested here (previous research has show mixed species to be retained)
Dose response capability	Yes	Yes	Yes	Require multiple Sorbarods
Ability to distinguish antimicrobial activity	Yes	Yes	Yes	No
Testing time	With frozen inocula – 24 hours	Species dependent ~5days	1 Week	2 Days
Resource required	Microplate reader	CDFF system	Microplate reader	GC system
Other testing endpoint capability	Substantivity (see chapter 6)			VSC test capability
Imaging revealing biofilm formation	Yes	Yes	No	Yes

The CDFF model system has been widely used in oral biofilm development and research (McBain *et al.*, 2003; Metcalf *et al.*, 2006; Pratten *et al.*, 1998; Vroom *et al.*, 1998; Wilson, 1990). In this evaluation it was shown that against developed biofilms CHX was the most antimicrobial, with zinc chloride being the least. Using CLSM, developed biofilms from the CDFF and Bioplate exhibited structural motifs of dense matrix and channels, similar to what has been reported previously (Hope and Wilson, 2003).

As a peer reviewed screening model, the MBEC system is the most similar to the developed Bioplate model as part of this thesis, in that they both utilise a 96-well microtitre plate. As in the CDFF model, the same pattern of antimicrobial activity was found in that CHX>triclosan>zinc chloride. However, during the analysis, it was found, across all MBEC plates used, that the MFU was lower than anticipated; this is a potential sign that the bacteria are not attaching and subsequently developing into a biofilm. Indeed, this was confirmed with the CLSM imaging, which confirmed the lack of biomass on the pegs. This model was not originally designed for use with oral organisms; therefore concerns exist for the use of this model with these organisms. The concern arises from growing oral biofilms on non-orally relevant surfaces, such as polystyrene for the MBEC pegs. It has been shown that the chemical and physical properties of the underlying substratum can affect the development of the conditioning film, including physiochemical surface properties, composition, density and configuration (Fletcher, 1976; Strevett and Chen, 2003). It has also been observed that oral bacteria can detach from a surface due to a cohesive fault

within the conditioning film (Busscher *et al.,* 1995). A second possible reason for the reduction in oral bacterial attachment to the pegs may be related to the findings from Parahitayawa (2006), who found that an increase in attachment of *Candida krusei* to the pegs of the MBEC, in comparison with *C. albicans*. Previous studies have shown, *C. krusei* to be more hydrophobic than *C. albicans*, thus having a greater propensity to attach to those surfaces (Samaranayake et al., 1995). Previous studies using ozone on dentine showed that modifications on the surface hydrophobicity prevented *S. mutans* from attaching (Knight *et al*., 2008). More recently, Samot *et al*. (2011) found that using an ATCC strain of *Lactobacillus casei* was unable to attach and subsequently develop into a biofilm on glass. Highlighting the importance of appropriate substratum when developing model systems. In another study (Grivet *et al*., 2000) identified, using hexadecane and water contact angles, that *S. mitis* was hydrophobic. It should be noted that in the same study, the authors also found, *S. mutans*, *S. sanguinis*, and *S. oralis* more hydrophobic than *S. mitis*. In this evaluation these organisms were able to develop biofilms on hydrophobic mental dental alloys; unlike *S.mitis.* Thus, the degree of hydrophobicity of the underlying substratum is of importance. Therefore, understanding the degree of hydrophobicity of the MBEC pegs and the test organisms, may help understand why there was poor biofilm formation.

Copper and zinc ions have been shown previously to be effective at removing VSCs (Jonski *et al*., 2004; Rolla *et al*., 2002), and are currently used in a number of oral care formulations (Rolla *et al*., 2002; Young *et al.*, 2001). The longevity of the effect of copper and zinc on VSC concentrations (to approximately 30 min-

post dosing despite a constant flow of fresh medium at 36 ml/h post-dosing) suggested that the test actives demonstrated a degree of inherent substantivity to the biofilm or else had an impact on bacterial viability or metabolic activity as well as chemically neutralising VSCs. Both metals demonstrate some antibacterial efficacy; however the minimum inhibitory concentration against *Streptococcus mutans* has been determined to be higher that the concentration required showing a chemical neutralisation effect (data not shown).

In contrast to metal ions, biofilm exposure to chlorhexidine resulted in a transient increase in VSC levels (Figure 77). Even 60 min post-treatment, biofilms were generating VSCs at baseline levels. This somewhat unexpected observation could be the result of chlorhexidine disrupting the bacterial cell wall and promoting the release of enzymes into the system that may still be available to convert amino acids to VSCs. This hypothesis requires further study to confirm, nevertheless, the data reported here would seem to match the *in vivo* situation. Thus, a recent clinical study demonstrated that brushing with a dentifrice containing the antimicrobial agent triclosan resulted in a transient increase in malodour when assessed using GC of mouth air (Newby *et al.*, 2008). In contrast, zinc-ion containing pastes afforded an immediate and statistically significant reduction in malodour. Thus, instead of investigating antimicrobial activity, this model was best placed to evaluate reductions in VSC's generated by malodour producing organisms.

During the test analysis utilising the Bioplate model exposed to chlorhexidine, triclosan and zinc chloride, the same pattern of antimicrobial activity was found in that CHX>triclosan>zinc chloride. In these studies the reported MFU, when tested against the negative control, water, was ~6000-7000 MFU, in the MBEC model this was ~500-600 MFU. These higher numbers suggest higher biomass formation and the presence of viable organisms. Indeed this was confirmed using the CLSM images, where biofilm can be seen developing around the hydroxyapatite crystals.

It should also be noted that testing time taken with the Bioplate, is less than what is required for the CDFF, MBEC and Sorbarod. Excluding initial culturing of the organisms, CDFF took 5 days biofilm development and 7 days for bacterial recovery and counts. The MBEC model took 5 days for biofilm development of anaerobic species and one day for evaluation. In comparison, the Bioplate took only two days to obtain test results.

In conclusion, it was found that the Bioplate model was able to evaluate antimicrobial test agents and differentiate activities against a preformed mixed species biofilm, whilst taking less time per analysis in comparison as the peer reviewed CDFF, MBEC and Sorbarod. Utilising an orally relevant surface, the Bioplate model was able to support a representative biofilms to allow *in vitro* high throughput testing against antimicrobial actives. Table 17 compares the evaluated model systems to each other, to clarify key differences with each model system.

Due to the positive results found here, it was discussed if there could be further possibilities of the Bioplate model. The desire to supplement antimicrobial activity with increased substantivity, akin to that seen with CHX is very desirable for oral health companies; however *in vitro*, high throughput methodology for specifically investigating substantivity is scare. Therefore the purpose of the next chapter is to evaluate and understand if modifying the Bioplate methodology to a high-throughput novel model to screen substantivity and the antimicrobial effectiveness of test actives.

CHAPTER 6: Utilising the developed *in vitro* biofilm model to generate a novel substantivity model.

6.1 Introduction

The search for new antimicrobial agents for improved plaque control requires that appropriate screening models be put in place. In the development of biofilm model systems, the use of relevant substrata is required to ensure that both the developed pellicle and subsequent biofilm mimic those found in the natural environment. The survival of microorganisms in various environments is dependent on their ability to attach/adhere to surfaces and subsequently develop into a biofilm. By modifying the surface to which the organisms attach, with substantive compounds, the ability of an anti-biofilm agent to reduce or prevent bacterial colonisation on these surfaces can be quantified. It is hoped that such a model will allow researchers to differentiate modes of actions between traditional antimicrobials and substantive/surface modifying compounds. In the previous chapter, a model system was discussed which investigated the antimicrobial efficacy of test agents against a preformed biofilm. The aim of this chapter was to develop a rapid screening system to identify novel antimicrobial agents with inherent substantive properties and subsequently prevent biofilm development for use in oral care.

A number of models exist which have been used to investigate the efficacy of substantive antimicrobial agents, such as flow cells (Busscher *et al.,* 2008), human teeth (Rasimick *et al.,* 2010) and dentine disk (Carrilho *et al*., 2010) and *in vivo* analyses (van der Mei, 2006), whilst all these model systems are appropriate for the study of substantivity, due to current methods they are not seen as appropriate

for high through-put screening of novel actives due to the long set-up and testing time.

Most oral care products specifically designed for the reduction in disease states, contain only a few choice actives, including chlorhexidine, triclosan and CPC. This review will focus primarily on the efficacy of CHX and triclosan, due to their differing modes of action in the oral cavity; CHX and CPC have very similar modes of action. Chlorhexidine (1,1'-hexamethylene-bis-5(4-chlorophenyl) bisguanide) is a broad spectrum, cationic antimicrobial with the chemical structure in Figure 81.

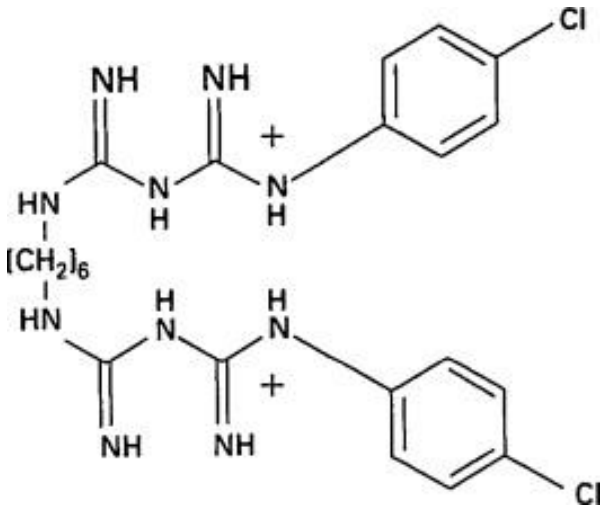

Figure 81 Chlorhexidine chemical structure

Due to its cationic nature, more specifically the hydrogen clustering, this gives rise to directionality of attachment, exposing the chloride ions out from the surface to the environment. CHX efficacy is well studied as a two-pronged mode of action; antimicrobial and retention on a biological surface, (Löe and Schiøtt,

1970; Mandell, 1994; Carrilho, et al., 2010). Indeed searching on PubMed using the search terms “antimicrobial AND chlorhexidine” identifies 5770 publications (as of June 2011). Unfortunately, as well studied as the benefits of CHX are, so are its side effects, such as tooth staining and modified taste perception (Addy and Moran, 1995; Nathoo and Gaffar, 1995). Again, due to its cationic nature, CHX gives rise to an increase in attachment of dietary chromagens (Addy et al., 1991) developing into a tenacious stain that can only be removed by the dentist.

Triclosan (2,4,4’trichlor-2’-hydroxydiphenyl ether) is a chlorinated bisphenol antimicrobial, the chemical structure is seen below in Figure 82.

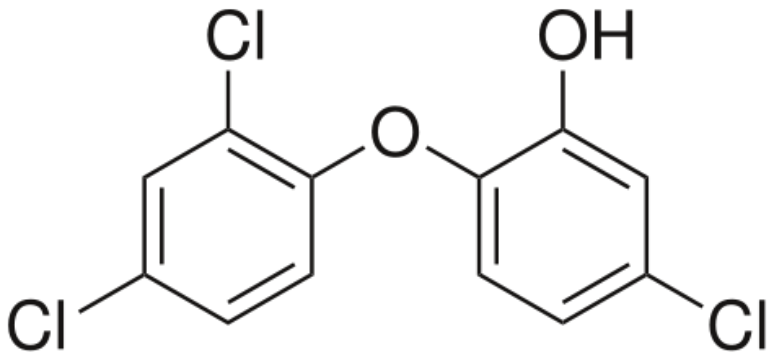

Figure 82 Triclosan chemical structure

Its antimicrobial activity has been attributed to the inhibition of the NADH enoyl-reductase enzymes of type II fatty acid synthase leading to bacterial cytoplasmic membrane damage and subsequent leakage (McMurry, 1998; McMurry, 1999). However, it is recognised that this cannot be the only pathway affected, however, no other route has been elucidated (Russell, 2004). Triclosan containing

toothpastes have been shown to be less effective, than when formulated in the presence of a retentive polymer (Gaffar *et al*., 1990; Renvert and Birkhed, 1995). In this instance, gantrez an anionic poly(methyl vinyl ether-co-maleic anhydride co-polymer is added to aid in the retention of triclosan to the surface to increase its substantivity to help increase its antimicrobial activity.

The desire to supplement antimicrobial activity with increased substantivity still exists, while reducing / removing any of the negative side effects, such as staining. The development of a daily use substantive agent for continual antimicrobial activity would certainly be of interest to both oral care companies and consumers alike. Indeed the commercial success of Colgate Total Care 12-hour toothpaste containing triclosan and gantrez are testament to that desire.

Having the capacity to evaluate and screen for such actives are paramount for the optimisation and progression to more clinically relevant *in vivo* models. The purpose of this research was to evaluate and understand the potential to modify the developed *in vitro* biofilm model as a novel model to screen substantivity and antimicrobial effectiveness of test actives. This development work utilized CHX and triclosan, known antimicrobial actives, with differing substantivity profiles.

6.2 Materials and methods

6.2.1 Minimum Inhibitory Concentration

This *in vitro* method was used to evaluate the inhibitory action of chlorhexidine and triclosan by the determination of their minimum inhibitory concentration. A series of dilutions of the test agent in a broth medium was prepared with the addition of an indicator dye and inoculated with *S. mutans* NCTC 10449. The method relied on a colour change in the pH indicator dye from red to yellow as the bacteria grow and produce acid as a result of their metabolic activity.

S. mutans was recovered from storage and plated on TSA and incubated at 37°C for 48 hours aerobically. After incubation a number of representative colonies were removed and placed in R+P, this was subsequently vortexed to completely suspend the organisms. A stock suspension with an absorbance between 0.3 - 0.4 at 600 nm (to equate approximately 10^8 cfu/mL). One millilitre of the stock suspension was added to 19 mL of double strength (DS) BHI with 0.01% w/v phenol red (Sigma) indicator to generate a suspension of approximately 10^6 cfu/mL. Double strength BHI was prepared by adding 74 g BHI broth (Oxoid) and 0.1 g phenol red indicator (Sigma) to 1 L deionised water and autoclaved at 121°C for 15 minutes.

Chlorhexidine solution was prepared by adding 0.1 mL of 20% v/v CHX (Sigma) to 9.9 mL sterile deionised water in 30 mL sterile containers (VWR) to generate a 0.2% v/v stock solution. Triclosan test solution was prepared by adding 0.5g of

Irgasan (Sigma) to 10 mL propylene glycol (Sigma) and left overnight in 37°C incubator to aid solubilisation. Once completely solubilised, 0.5 mL of the 5% w/v stock solution was added to 9.5 mL sterile deionised water to create a 0.25% v/v stock solution.

Using a 96-welled microtitre plate, 100 µL of diluting diluent; water in the case of CHX, and 5% propylene glycol in the case of triclosan, was added to each well from column 2-12. One hundred microlitres of the test samples were added in duplicate to row A and B (CHX) and C and D (triclosan), this was added to both column 1 and 2 in both occasions. Using a multi-channel pipette, the samples in column two were mixed by aspiration (x3) and 100 µL transferred to column 3. This method was repeated across the plate to generate a doubling dilution series, from the last well 100 µL was removed and discarded. To each of the 96-wells 100 µL of the inoculum suspension in DS BHI + 0.01% v/v phenol red was added. The plate was covered with titre-tops (Thermo Scientific, Waltham, MA, USA) to prevent dehydration and incubated overnight aerobically at 37°C on an orbital shaker set at 200 rpm. Controls were carried out to ensure test organism viability, no residual antimicrobial activity with respect to dilution diluents, and to ensure no test agent interference with phenol red indicator. MIC was recorded as the last well that showed no colour change as seen in Figure 84.

6.2.2 Bioavailability Model Developments

The model system utilises a 96-well microtitre plate coated with HA using the methodology described in chapter 2, and subsequently coated with a mucin-containing artificial saliva pellicle.

S. mutans NCTC 10449 was grown from stock culture, generated as in chapter 2 and plated on TSA and incubated at 37°C for 48 aerobically. Using a sterile loop a representative colony was removed and inoculated into 20 mL pre-warmed BHI broth, this was subsequently incubated overnight at 37°C with shaking at 200 rpm. After incubation, one mL of the inoculated BHI was removed and added to fresh pre-warmed 20 mL. This was incubated at 37°C for 3 hours to obtain cultures in early exponential phase growth.

Test solutions of chlorhexidine digluconate (substantive compound) and triclosan (non-substantive compound) were prepared at concentrations ranging from 1 to 0.001%. The concentrations tested were 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004, 0.002, 0.001% v/v. Sterile deionised water was used as a negative control. Two hundred microlitre of the test agents and controls were added to a blank 96-welled microtitre plate, where each concentration was added to columns, thus n=8 for each sample.

Artificial saliva containing mucin was manufactured (2.5g porcine stomach mucins, Sigma; 0.35g sodium chloride, VWR; 0.2g potassium chloride, Sigma; 0.2g calcium chloride dehydrate, VWR; 2.0g yeast extract, Oxoid; 1.0g lab lemco

powder, Oxoid; 5.0g proteose peptone, Oxoid; 1.25mL 40% Urea solution, Oxoid; 1L deionised water) and sterilised. Once cooled, 200 µL were added to each well in two, 96-welled microtitre plates. These plates were then incubated at 37°C for 30 minutes to allow the development of a pellicle over the hydroxyapatite coating as seen in Figure 83. Using a Nunc-Immunowash (12 manual plate washer fed with TSB and attached to an effluent bottle via a vacuum pump) the artificial saliva was removed, however the wells were not washed.

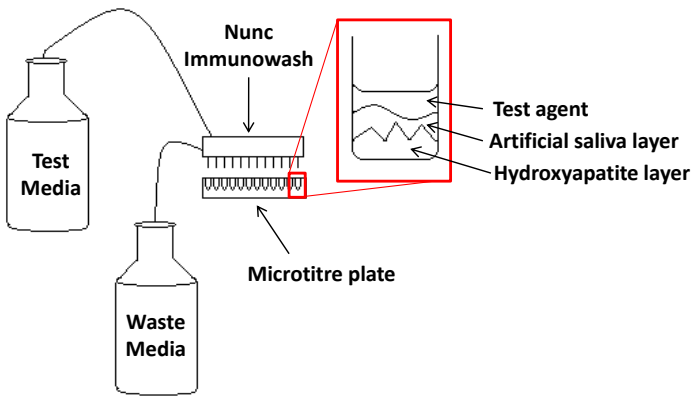

Figure 83 Schematic of bioavailability method, highlighting the sequence of deposition of the hydroxyapatite, artificial saliva and test agent.

Using a Transtar pipette (Costar), the previously plated 200 µL prepared test agent was transferred to the artificial coated HA-coated microtitre plates. Each plate was then incubated at 37°C with shaking at 200 rpm for 2 minutes, replicating recommended treatment timings for most oral care products. Using the Nunc-immunowash again, the test samples were removed and each well washed twice with BHI, to remove any unbound test agent. To one plate was added 20 µL of BHI + inoculum and 80 µL of 10 v/v AlamarBlue™ in BHI to each well. This

plate was incubated with shaking at 37°C and fluorescence read every 20 minutes for 300 minutes. Fluorescence was measured using a 530 nm excitation filter and 590 nm emission filter. Results are recorded as mean fluorescence units (MFU).

To the second plate 20 µL BHI + inoculum was added to 180 µL of BHI. This plate was incubated overnight at 37°C aerobically without shaking. After which, using the Nunc-Immunowash, the broth was removed from each well and washed twice with sterile BHI to remove any loosely attached and unbound organisms, leaving the developed biofilm. After the second wash the wells are left empty and to each well was added 200 µL BHI with 10% v/v AlamarBlue™. The plate was subsequently incubated for a further 30 minutes aerobically at 37°C with shaking at 200 rpm. Fluorescence was measured using a 530 nm excitation filter and 590 nm emission filter. Results recorded as MFU.

6.2.3 Bioplate Testing

Using the methodology developed previously in chapter 2, using donated saliva, following appropriate methodologies for recovery. Each well of a HA coated 96-well microtitre plate was inoculated with 20 µL of the freshly prepared bacterial culture. Subsequently, 180 µL of sterile BHI was added to each well, plates were then incubated aerobically for 24 hours at 37°C without shaking.

When analysing the antimicrobial activity of test agents, the plates containing the test agents were prepared in advance to be readily available for use. Test solutions of chlorhexidine digluconate and triclosan were prepared at

concentrations ranging from 1 to 0.001% v/v. These were prepared at the following concentrations 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004, 0.002, 0.001% v/v. Sterile deionised water was used as a negative control. To each column on an uncoated 96-well microtitre plate was added 200 µL of the test agent. This generated 8 replicates in one column for each test agents.

Taking an inoculated Bioplate that has been incubated overnight, exhausted media from each well was removed using a Nunc-Immunowash. Wells were washed (x2) using the Immunowash to remove unattached or loosely bound bacterial cells, thus leaving the wells of the Bioplate empty except for the developed biofilm. Using a 96-Transtar pipette and the uncoated, test agent containing 96-well plate prepared previously, which contains 200 µL of the test agents in columns, the test agents were transferred to the now empty Bioplate and incubated at 37°C for 2 minutes while shaking. Subsequently, using the Nunc-Immunowash each well was washed (x2) with sterile TSB to remove traces of the test agent leaving the Bioplate empty. Using an 8x multichannel pipette, 200 µL of sterile TSB was added to each well on the plate and re-incubated for 4 hours at 37°C. To measure the viability of the biofilm, a 10% v/v solution of AlamarBlue™ in TSB was prepared. After the second incubation, the Bioplate was removed from the incubator and each well washed twice with sterile TSB. Using an 8x multichannel pipette, 200 µL of the 10% v/v AlamarBlue™ solution was added to each well. The plate was further incubated for 30 minutes at 37°C aerobically with shaking. Fluorescence was measured using a microtitre plate reader using a 530 nm

excitation filter and a 590 nm emission filter. Results were recorded as mean fluorescence units (MFU).

6.3 Results

6.3.1 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of triclosan was found to be 20 µg/mL (0.0020%). The MIC of CHX was found to be 2 µg/mL (0.0002%). Figure 84, represents the colour change seen with phenol red indicator. All controls used in this methodology performed as expected.

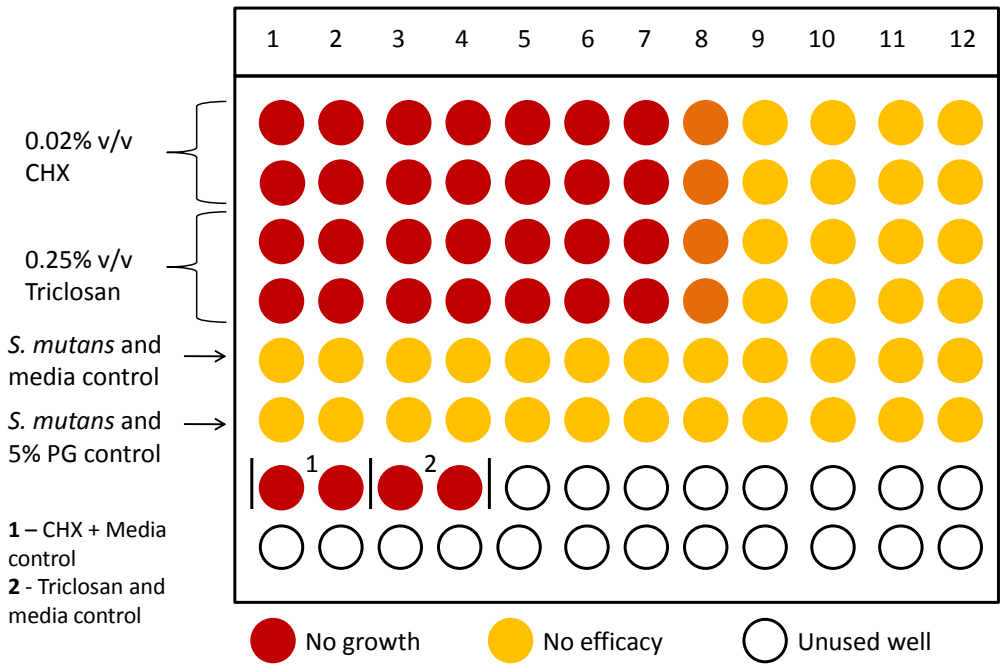

Figure 84 Diagrammatic representation of colour change resulting from the phenol red pH indicator in the MIC methodology. The MIC is reported as the last well with no growth. CHX and triclosan tested in duplicate.

Initial studies were carried out (data not shown) using a lower concentration of triclosan, however the MIC could not be determined using this methodology, therefore, the starting concentration was increased to locate the MIC, using this model.

6.3.2 Bioavailability

Figure 85 shows the results for the CHX dose response using the new bioavailability model. This result shows the antimicrobial efficacy of bound CHX. Noting that the wells were washed post-CHX treatment.

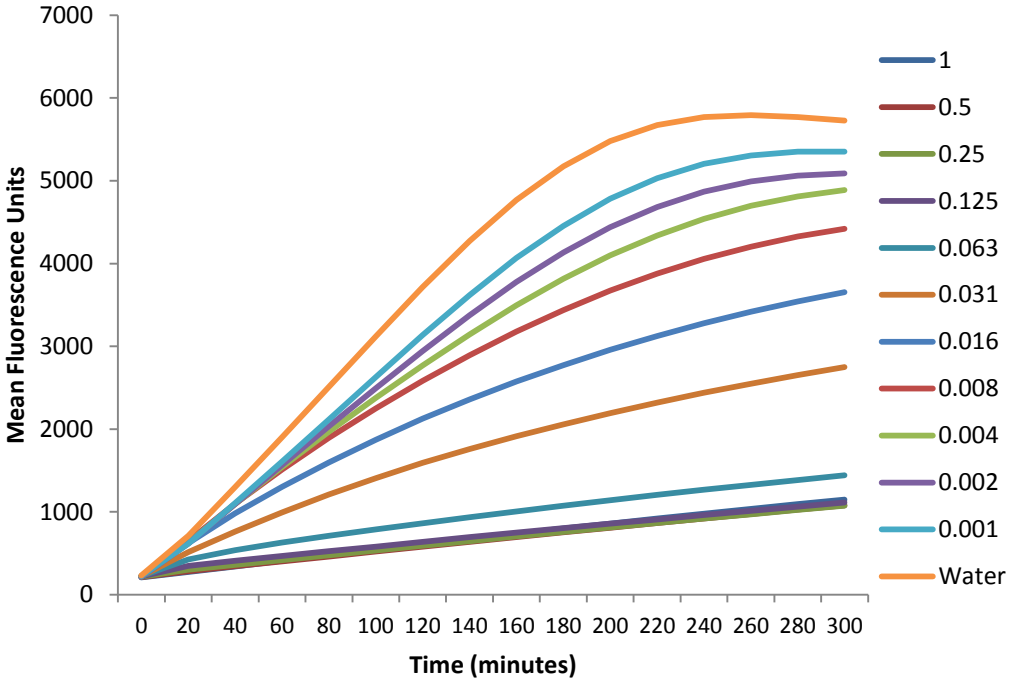

Figure 85 The effect of chlorhexidine [% v/v] dilutions on the prevention of biofilm development on pellicle coated HA surfaces after a two minute contact time (n=8). Bacterial viability was monitored for 5-hours post dosing, readings taken every 20 minutes.

It is seen from the water control that there was no inhibition of bacterial growth, metabolism or viability as measured by AlamarBlue™. At the higher concentrations of CHX >0.0063% v/v it was found a clear reduction in viability a seen in Figure 85. All the concentrations gave a clear dose response.

However, the same dose response was not seen in with the organisms incubated for 24 hours and allowed to attach to the treated surfaces, where a 0.008% v/v CHX solution was required to prevent biofilm development 24-hours post-dosing Figure 86.

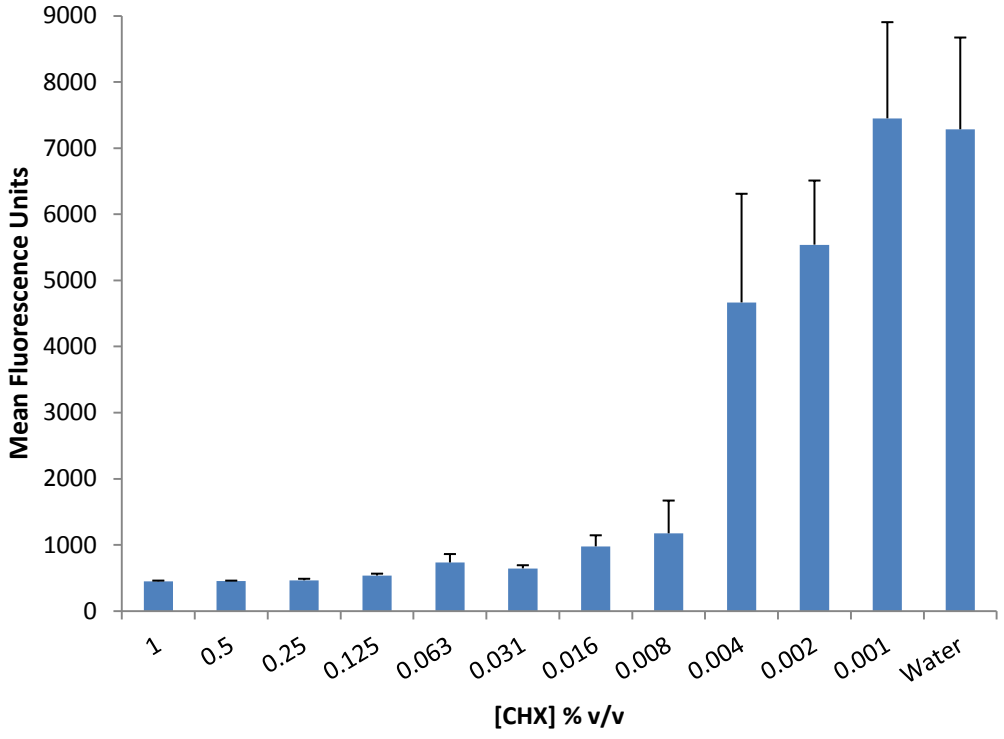

Figure 86 Viability of biofilms formed on 2 minute treated HA coated surfaces with CHX dose response, 24-hours post-dosing (n=8).

The effectof CHX on 24-hour biofilms post treatment of the surface was analysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$.

Treatments with the same letters are not significant. Treatments 1-8 (0.008-1% CHX), where treatment 1 was the positive 1% CHX control, in this study. Treatments 11 and 12 are statistically similar, where treatment 12 is the negative control water. Treatments 9 and 10 are distinct from both the positive and negative controls.

Method: 95.0 percent LSD

Treatments	Count	Mean	Homogeneous Groups
1	8	450.0	A
2	8	451.5	A
3	8	462.875	A
4	8	535.375	A
6	8	643.25	A
5	8	732.625	A
7	8	976.25	A
8	8	1173.5	A
9	8	4665.13	B
10	8	5538.38	C
12	8	7283.5	D
11	8	7448.63	D

There was no inhibition of cell viability or biofilm development on surfaces treated with levels 0.004% v/v or below, 24-hours post-dosing. There was no reduction in cell viability with the water control. In Figure 85, 0.008% v/v CHX was shown to retain some viability, however, in the biofilm development stage of this model there was no viability left or signs of biofilm development on the CHX modified surface above that concentration.

In contrast to chlorhexidine, when treated with a triclosan dose response, there was no inhibition of bacterial viability across the concentrations tested, as seen in Figure 87.

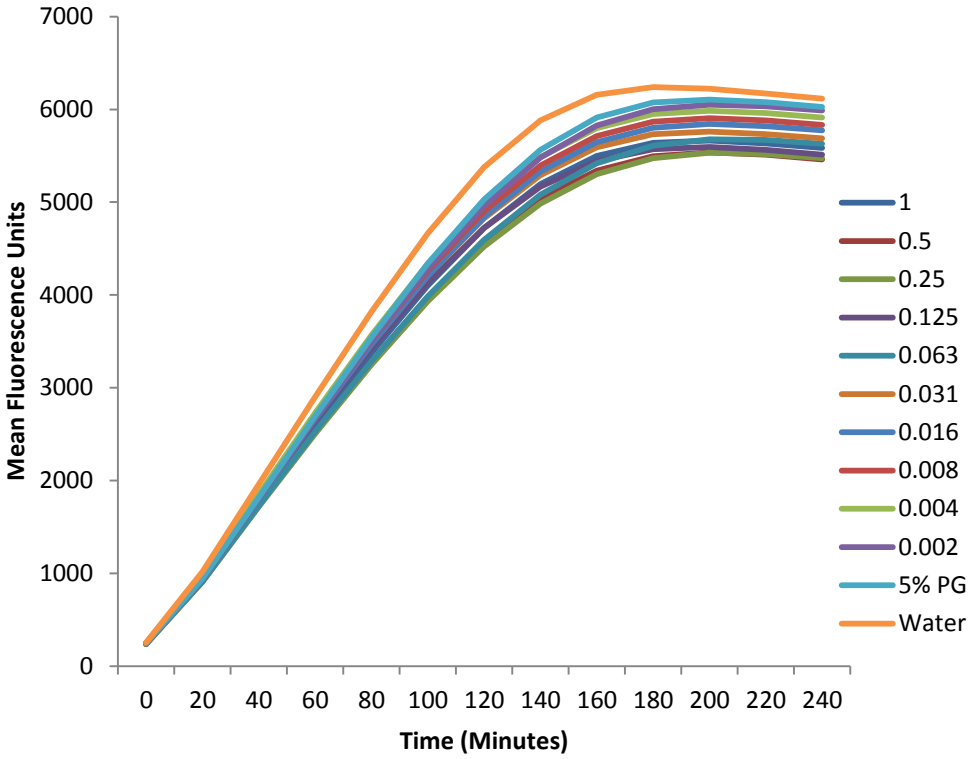

Figure 87 The effect of triclosan [% v/v] dilutions on the prevention of biofilm development on pellicle coated HA surfaces after a two minute contact time (n=8). Bacterial viability was monitored for 4-hours post dosing, with readings taken every 20 minutes. Experiment stopped at 240 minutes due to plateau.

There was found to be no reduction in viability with either the water or 5 % v/v propylene glycol negative control. Five % v/v propylene glycol was added to this evaluation to ensure no antimicrobial or substantivity could be attributed to the triclosan solubilisation diluent. This study concludes that post-triclosan exposure and subsequent washing, no antimicrobial efficacy was retained at the surface in this model was found.

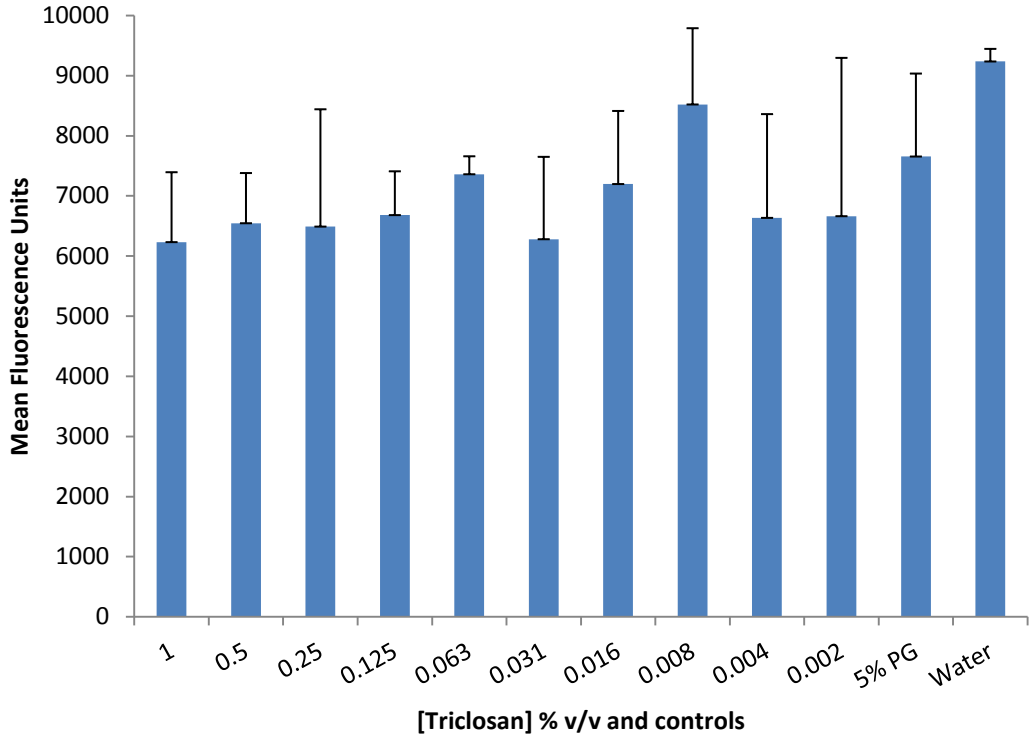

Figure 88 Viability of biofilms formed on 2 minute treated HA coated surfaces, 24-hours post-dosing (n=8) with triclosan dose response. All samples are statistically similar where p=0.0003.

When incubated overnight to allow biofilm development on the surface, it was found that there was no reduction in biofilm development or viabililty with a dose response with triclosan as seen in Figure 88, where p=0.0003.

6.3.3 Bioplate Results

A chlorhexidine dose response indicates that the concentration of chlorhexidine required to reduce fluorescence to background levels was 0.031% v/v, at a concentration of 0.004% v/v there is a clear split in efficacy from higher concentrations. However, a slight decrease in CHX antimicrobial efficacy can be seen from 0.016% v/v. There was no antibacterial activity at chlorhexidine concentrations below 0.004% v/v.

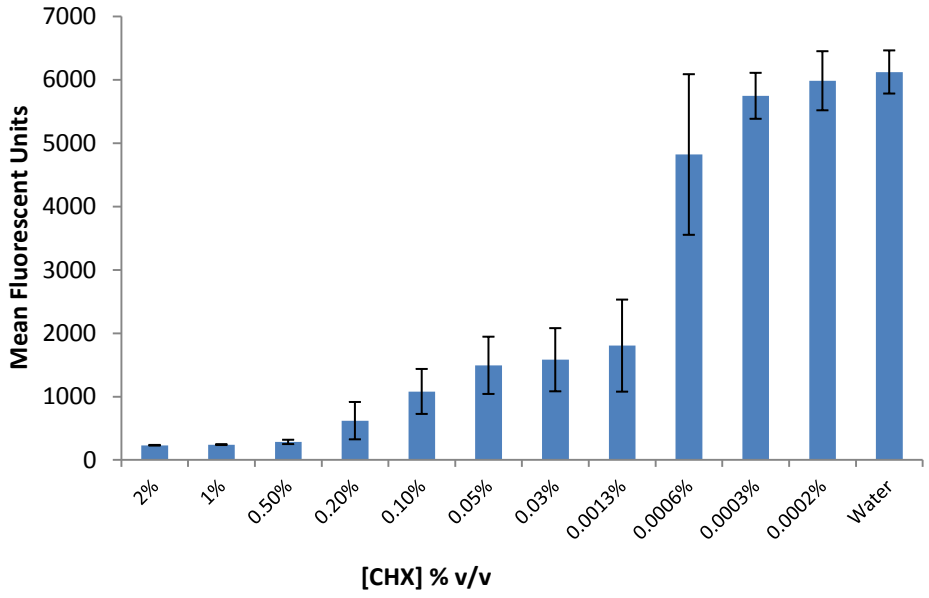

Figure 89 Viability of 24-hour preformed biofilms, grown on HA coated microtitre wells, using the developed Bioplate methodology, post 2 minute exposure to CHX dose response (n=8).

The effect of CHX on a preformed biofilm using the Bioplate methodology was aqnalysed using a one-way analysis of variance with a factor for treatment, where $p<0.05$. Treatments with the same letter are not significant.

Method: 95.0 percent LSD			
Treatments	Count	Mean	Homogeneous Groups
1	8	231.625	A
2	8	242.0	A
3	8	284.0	A
4	8	619.625	AB
5	8	1080.75	BC
6	8	1493.25	CD
7	8	1581.75	CD
8	8	1804.13	D
9	8	4820.88	E
10	8	5747.38	F
11	8	5985.38	F
12	8	6123.75	F

Treatments 1 – 8 (0.0013 – 2% CHX are not statistically sisimilar to each other, treatments 10-12 are statistically similar, where treatment 12 is the deionised water control. Treatment 9 is statistically distinct from the negative and positive control.

A triclosan dose response found that, in this model there was no antimicrobial efficacy across all the concentrations tested. The water negative control did not reduce biofilm viability and seen in Figure 90.

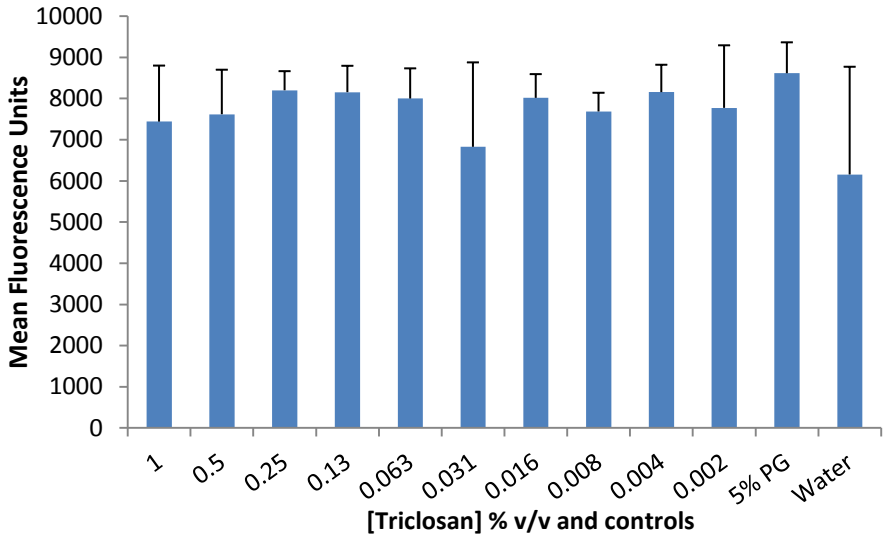

Figure 90 Viability of 24-hour preformed biofilms, grown on HA coated microtitre wells using the developed Bioplate methodology, post 2 minute exposure to triclosan dose response (n=8). Where p=0.0190

The effect of triclosan on a pre-formed biofilm, using the developed Bioplate methodology was analysed using a one-way analysis of variance with a factor for treatment, where $p=0.0190$.

The results from both studies show that in this model, where there is only a two minute exposure to the test agents that the model has the capacity, dependant on test agent under evaluations, to differentiate activity on the chemical's attributes. Either surface attached, expressing an antimicrobial effect, such at the substantivity seen with CHX.

6.4 Discussion

Due to tightening regulations, the numbers of new and novel antimicrobials being developed and subsequently approved is diminishing. This model allows the evaluation of 'traditional' antimicrobials and other formulation excipents, to understand any possible substantivity effects. The model also allows for the evaluation of excipients in the presence of antimicrobials to increase substantivity. This high through-put screening model gives confidence of success prior to entering into expensive *in vivo* clinical studies.

The test agents utilised in this initial study, CHX and triclosan are well known oral antimicrobial compounds with differing substantivity profiles. Guggenheim (2001) found that a 1 minute exposure to a 0.2% w/v CHX solution with 10% ethanol resulted in a decrease in the recovery of CFU by ≥2 order of magnitude. This is a greater effect than what was seen using 1% w/v triclosan in his model, showing an increase in efficacy of CHX in comparison with triclosan. This study here also found CHX to be more antimicrobial that triclosan, in the initial MIC studies. Interesting a recent study carried out by Ledder *et al.* (2010), shows triclosan to be the most antimicrobial agent in comparision to stannous fluoride (0.45% w/v), zinc lactate (2.5% w/v), and more surprisingly sodium dodecyl sulfate (1.00% w/v). Indeed, Jenkins (1991) reported a small scale clinical evaluation where SDS was more effective at reducing salivary bacterial counts, more effectively that triclosan, but not as effectively as CHX. However, it should be noted that the model utilised by Ledder *et al.* (2010), uses a 20 minute exposure time. This is not reflective of in use recommendations for these

compounds in oral care products. This bioavailability model, as show in Figure 86 and Figure 88, has the capacity to investigate the substantivity of test agents after only a two minute exposure; this exposure time can easily be modified to suit the individual evaluations of the research.

As discussed previously in the introduction, the inherent hydrophobicity of the test substrate will affect the bacterial attachment and subsequent biofilm formation. A recent study carried out by Wang et al., (2011) showed using three organisms of differing hydrophobicity to attach to surfaces of differing hydrophobicity using *Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas putida* attaching to glass and octadecyltrichlorosilane modified glass. This study highlights the principle that depending on the substrate, changes in the bacterial attachment could be made. In this study the attachment to hydrophilic glass the attachment was found to be *E. coli*> *P. putida*> *P. aeruginosa*. On the hydrophobic octadecyltrichlorosilane glass bacterial attachment trend was *P. aeruginosa*> *P. putida*> *E. coli*. It has been reported that the development of a conditioning film on the test substrate in the initial step for biofilm formation. Changes in the substrates can modify the conditioning film structure (Loeb et al., 1975). Mittelman (1996) further discusses the effect of changes in the conditioning film structure in biofilm attachment and subsequent biofilm development.

With further research and the development of a mixed microcosm, this substantivity screening model would have the capacity to monitor the test agent

ability to prevent biofilm growth in the presence of mixed species and co-aggregated bacterial communities. Therefore, using this assay, test agents can be ranked by, not only their ability to kill, but also their ability to prevent biofilm formation.

Discussion

As previously stated, the effects of oral biofilms on the economic, social and personal burden can be challenging. It has been reported by the World Health Organisation, that oral disease is the 4th most expensive disease to treat in industrialised countries, with 60-90% of school children globally suffering dental caries (WHO Factsheet 318, 2007). In 2006, in England alone, the National Health Service spent almost £2 billion in dentistry for dental treatments and disease prevention, equating to around £40 per head of population (NHS Report, 2008).

The human oral cavity contains a number of different surfaces each providing a unique physiological environment for bacterial colonisation and biofilm (plaque) development. The physical removal of plaque bacteria by brushing is the main contributor to the maintenance of healthy teeth and gums. Increasing the numbers of bacteria removed and/or killed during brushing is an approach favoured by many healthcare companies via the addition of effective antimicrobial agents to oral care products. Therefore, there is an ongoing need to identify novel antimicrobial agents that can be incorporated into oral healthcare products e.g. dentifrices and mouthrinses. Thus, having the ability to efficiently and effectively screen actives against orally relevant biofilms is a key challenge for industry to help address patient's and consumer's oral care needs.

As previously stated, and to reiterate, when developing an orally relevant biofilm model, the closer we can be to the *in situ* scenario, the more representative the results will be of the *in vivo* effect. The search for new antimicrobial agents for

improved plaque control requires that appropriate screening models be put in place. Key criteria for these models include;

- Results should be predictive of clinical outcome
- Orally relevant organisms (including mixed species if possible)
- Bacteria should be present in biofilms
- Short, relevant contact time should be used
- Reproducible methodology
- Rapid and high throughput (~800 screenings/year) capacity

The overall aim of this research was the development of a reproducible 'off-the-shelf' assay to demonstrate the antimicrobial efficacy of test agents against orally relevant biofilms. All of the parameters described above were considered in order to develop a robust and reproduciable method for the evaluation of antimicrobials, such that a Consumer Healthcare company could have a high volume screening methodology for the investigations into novel test actives for use in oral care products. It is envisaged that the model could be modified to produce different microbiota associated with oral diseases including the tongue, supragingival and subgingival (in particular those responsible for gingivitis) populations. The research carried out here focused primarily on the generation of representative supragingival microbiota. Many *in vitro* oral biofilm studies require the development of orally relevant plaque. However, many studies focus primarily on single-species biofilms which do not take into consideration the multi-species interactions which occur in plaque (Adams *et al*., 2002; Luppens *et al*., 2008; Palmer *et al*., 2001). *In vitro* biofilm structure is determined by available

nutrients, the substratum of the model, the organisms present and the incubation conditions. Changes in any one of these parameters will affect bacterial growth and biofilm development, thus affecting biofilm structure. Therefore, the initial aim was to grow orally relevant biofilms on a hydroxyapatite (HA) coated microtitre 96-well polystyrene plates.

Initial evaluations revealed changes to the incubation conditions and media could impact bacterial viability negatively. Once the methodology was optimised to retain viability, it was found that the system still lacked reproducibility, thus a move to a defined inoculum from salivary derived inoculum was investigated. Two parallel studies were undertaken that investigated the use of a defined inoculum based on the work by Marsh (1985) to that of pooled donated human saliva. Initial molecular analysis suggests the existence of uncultured bacterial clones within the donated saliva, which may add to biofilm complexity, therefore, more closely reflecting *in vivo* biofilms. However, it has been shown previously that the complexity of the microbiota is lost when using *in vitro* systems (Pratten *et al*., 2003) and there are possible problems when using such inocula in maintaining reproducibility. Therefore, to further aid in the refinement of the model a defined inoculum was investigated. It has been shown that the inocula should contain precise quantities of each strain in order to obtain a final biofilm with a representative consortium containing proportions of bacteria as would be seen *in vivo* (Pratten *et al*., 1998). Furthermore, it is envisaged that it would be possible to change the composition of the mixed consortia in order to replicate different oral microbiota. Such studies on shifting the microbiota with dual and

microcosm oral biofilms have been successfully carried out previously (Dalwai *et al*., 2006). The initial findings showed that by using the defined inocula first described by McKee (1985) a representative biofilm could be created in the microtitre plates.

One of the key aspects of this model to be successful is to permit faster throughput, and having to grow up each bacterial species prior to testing would be labour intensive and cumbersome. Therefore, the defined inoculum was evaluated for stability up to three months at -80 C in either 5 or 10% v/v glycerol incubated either in an Eppendorf tube, or on the Bioplate. The key finding was the retention of the mixed species viability for one month in 10% v/v glycerol. Five percent glycerol, showed little retention of viability over the three month stability test, in both the Eppendorf tube and the HA coated microtitre plate. The method permits faster turnaround for testing, as the initial bacteria growth and set up has been removed. This is a saving of 7 days set up before each analysis. To truly offer an 'off-the-shelf' model system it would have been preferred that the organisms remained viable on the HA coated plate. Further analysis looking at larger sample volumes during storage and increased glycerol concentration, may still offer opportunities to deliver pre-inoculated HA coated microtitre plates from storage.

Previously, Mariscal *et al.* (2009) utilised resazurin dye as an indicator of bacterial respiration. The author reported correlation with bacterial numbers and showed that it is possible to estimate bacterial counts via the generation of initial growth curves. It should be noted that this method was recommended if the

bacterial numbers were between $1x10^3 – 1x10^8$ CFU for an estimate of the CFU to be made after 5 hours. However, this study utilised single species. The biofilm model developed as part of this PhD utilised mixed species biofilms to be more representative of what would be found in the oral cavity. These differences in growth rates and respiration of these different organisms make this method of correlation between cell count and MFU unreliable in this biofilm model.

To further investigate the model system it was evaluated alongside peer reviewed model systems currently available for modelling biofilms found in the oral cavity, such as the CDFF, MBEC and Sorbarod. The comparison focussed on comparing a number of recognised oral care antimicrobial agents, chlorhexidine, triclosan and zinc chloride/acetate. Table 18 provides a summary of the models in comparison with one another. The CDFF concept was originally discussed in 1974 by Atkinson and Fowler, focussing primarily on a microbial film fermenter. Utilising this concept and building on research carried out by Coombe (1981), further model developments by Peters and Wimpenny in 1988, resulted in a model that would allow the development of replicate biofilms of a defined thickness. This model permits the development of biofilms between 50-600 μm thick due to the biofilms being grown in recessed pans.

Table 18 Comparison of model systems for oral biofilm development and subsequent antimicrobial analysis.

	Developed Bioplate	**CDFF**	**MBEC**	**Sorbarod**
Capable of growing mixed species biofilms	Yes	Yes	Yes	Yes
Peer reviewed with respect to oral bacteria	No	Yes	No	Yes
Orally relevant surface	Yes	Yes	No	No
Time to evaluate	With frozen inocula – 24 hours	Species dependent ~5days	1 Week	2 Days
Number of agents per evaluation	12	7	12	1
Replicates	8	5 biofilms per pan	8	Dependent on set up – Three possible
High throughput	Approx 6 plates daily	No	Approx 6 plates daily	No
Availability	Inhouse manufactured-raw materials easily available	Only at academic institutes	Commercially available	In house manufactured
Other testing capabilities	Substantivity – see chapter 6	VSC, longitudinal studies, intermittent pulsing of the antimicrobial or antibiotic	MIC	VSC analysis using GC
Dose response	Yes	Yes	Yes	Yes
Culture	Batch	Continuous	Batch	Continuous

This is a closed model system where the environmental gas, medium and inoculum can be controlled. The biofilms can be removed and evaluated for bacterial counts, architecture and antimicrobial efficacy of any test substances. The CDFF model system has been widely used in oral biofilm development and research (McBain *et al.,* 2003; Metcalf *et al.,* 2006; Pratten *et al.,* 1998; Vroom *et al.,* 1999; Wilson, 1990). The MBEC model was developed from the Calgary Biofilm Device (CBD) and developed by Ceri *et al.,* (1999). A key reason for utilising this methodology is that it is a peer reviewed, 96-well microtitre plate biofilm screening model. The MBEC model system utilises both the lid, which contains 96 pegs, corresponding to each of the 96-wells on the base of the plate. Once a test inoculum is added to the microtitre plate and the plate incubated at an appropriate temperature and time, biofilms will form on the pegs. The model was initially developed to investigate the antibiotic susceptibility of attached bacteria, whilst in a biofilm state.

During this evaluation it was shown that against developed biofilms CHX was the most effective antimicrobial, with zinc chloride being the least, this was seen across all model systems used. Interestingly, there was little evidence in this anyalsis, using CDFF, MBEC, or Bioplate of zinc chloride's antimicrobial activity, however in the Sorbarod model, zinc was effective at reducing VSCs. Using CLSM, developed biofilms from the CDFF and Bioplate exhibited structural motifs of dense matrix and channels, similar to what has been reported previously (Hope and Wilson, 2003). However, during the analysis, it was found, across all MBEC plates used, that the MFU was lower than anticipated; this is a

potential sign that the bacteria are not attaching and subsequently developing into a biofilm. Indeed, this was confirmed with the CLSM imaging, which confirmed the lack of biomass on the pegs. This model was not originally designed for use with oral organisms; therefore concerns exist for the use of this model with these organisms. The concern arises from growing oral biofilms on non-orally relevant surfaces, such as polystyrene for the MBEC pegs. It has been shown that the chemical and physical properties of the underlying substratum can affect the development of the conditioning film, including physiochemical surface properties, composition, density and configuration (Fletcher, 1976; Strevett and Chen, 2003). Having the capability to grow biofilms on a hydroxyapatite surface that reflects surface characteristics similar to enamel, is paramount for the development of orally relevant biofilms, AFM studies have shown that the development of biofilms on surfaces can be monitored, indeed in the limited studies carried out here, showed that the technology was capable of differentiating organisms merely settled to the surface and those actively attached.

It should also be noted that testing time taken with the Bioplate, is less than that required for the CDFF, MBEC and Sorbarod. Excluding initial culturing of the organisms, CDFF took 5 days biofilm development and 7 days for bacterial recovery and counts. The MBEC model took 5 days for biofilm development of anaerobic species and one day for evaluation. In comparison, the Bioplate took only two days to obtain test results, thus the desire to have a high thoughput model capable of ~800 actives a year can be met with this model system.

Taking the developed model further, it was investigated whether minor modifications to the Bioplate model would result in a rapid screening system to identify novel antimicrobial agents with inherent substantive properties and subsequently prevent biofilm development. The survival of microorganisms in various environments is dependent on their ability to attach/adhere to surfaces and subsequently develop into a biofilm. By modifying the surface to which the organisms attach, with substantive compounds, the ability of an anti-biofilm agent to reduce or prevent bacterial colonisation on these surfaces can be quantified. The desire to supplement antimicrobial activity with increased substantivity, akin to that seen with CHX is very desirable for oral health companies. A number of models exist which have been used to investigate the efficacy of substantive antimicrobial agents, such as flow cells (Busscher *et al.*, 2008), human teeth (Rasimick *et al.,* 2010) and dentine disk (Carrilho *et al*., 2010) and *in vivo* analyses (van der Mei, 2006), whilst all these model systems are appropriate for the study of substantivity, due to the methodologies they employ they are cannot be appropriate for high through-put screening of novel actives. Most oral care products specifically designed for the reduction in disease states, contain only a few choice actives, including chlorhexidine, triclosan and CPC. This evaluation focussed on CHX and triclosan, due to their differing modes of action in the oral cavity; CHX and CPC have very similar substantivity and antimicrobial profiles. The development of a daily use substantive agent for continual antimicrobial activity would certainly be of interest to both oral care companies and consumers alike. With further research and the development of a mixed microcosm, this substantivity screening model would have the capacity to monitor the test agent

ability to prevent biofilm growth in the presence of mixed species and co-aggregated bacterial communities. Therefore, using this assay, test agents can be ranked by, not only their ability to kill, but also their ability to prevent biofilm formation.

In conclusion to this thesis, two models have been developed

1. Utilising a preformed biofilm to evaluate antimicrobial activity.

2. Using a HA coated plate exposed to test agents to evaluate, substantivity to HA surface and the prevention of biofilm development on that surface.

Each model, allows for a high-throughput system to evaluate a number of test agents, utilising defined orally relevant organisms, thus ensuring the system repeatability. Both systems also offer the capability to control exposure time to the actives. This being of important when current oral health guidance show that mouthwashes should be used for 30 second to 1 minute, and toothpaste for 2 minutes (for adults).

Both these model systems have been successful trained to peer microbiologists at GlaxoSmithKline for the rapid and high-throughput of initial antimicrobial efficacy, where the organisms should be present in biofilms.

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Publications Resulting from this Thesis